RICE UNIVERSITY

Influence of Peroxisomal Import and Receptor Recycling on Peroxisomal Function

by

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A THESIS SUBMITTED IN
PARTIAL FULFULLMENT OF THE
REQUIREMENTS FOR THE DEGREE

Doctor of Philosophy

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HOUSTON, TX
DECEMBER 2010

ABSTRACT

Peroxisomes compartmentalize a variety of important metabolic reactions including fatty acid β-oxidation and the related process of IBA β-oxidation. Peroxisomal proteins are encoded by nuclear genes and must be post-translationally imported. A dynamic import process is vital for proper matrix protein localization and is dependent on the family of peroxin (PEX) proteins. The delivery and peroxisomal import of cargo from a loaded receptor, PEX5 or PEX7, is carried out by the early-acting peroxins, including PEX13 and PEX14, and receptor recycling is carried out by the late-acting peroxins, including PEX4 and PEX6.

In this thesis, I describe the use of double mutant analysis to differentiate early-acting and late-acting *pex* mutants by phenotypic and molecular analysis. I found that double mutants made with two early-acting or two late-acting *pex* mutants showed enhanced phenotypes in ß-oxidation and import defects. In contrast, defects of double mutants made with a weak early-acting mutant and a late-acting mutant were suppressed. Additionally, I found that receptor localization is central to proper peroxisomal function. My results suggest that when the receptor is not removed from the peroxisome, stabilized peroxisomal pores may be formed, perhaps impairing peroxisomal function due to leaching of peroxisomal contents. Together my data suggest that balance between import and receptor recycling is fundamental for peroxisomal function.

In humans, peroxisomal biogenesis disorders are most often caused by defects in late-acting peroxins. Peroxisomal defects occur in plants and humans as a result of the same lesions in PEX proteins. The understanding of how these late-acting defects can be ameliorated in plants, may inspire new approaches to human therapeutics.

ACKNOWLEDGEMENTS

I would like to thank Bonnie Bartel for being a wonderful mentor and teacher. She was always there to help, but let me learn on my own. She watched me fail and was supportive and encouraging. Then she watched me succeed and gave me the freedom to go wherever I could imagine. I will always be thankful to her for that gift.

Thank you to my coworkers for providing valuable insight and scientific discussion. I would especially like to thank Naxhiely Ramon for not only being an understanding coworker but also a good friend.

The biggest source of encouragement, support, and motivation came from my family. I would like to thank my mom, Marsha, has always been my soft place to fall, my sister, Allison, and brother, Eric, who inspire me to be a worthy big sister, and my husband, Loren, who has made me laugh through this journey. Lastly, I would like to dedicate this thesis to the memory of my father, Dr. John Ratzel. Rarely does brilliance come packaged in such a wonderful person. I truly know what excellence looks like because of him.

I would also like to thank my committee for their consideration and sharing their wisdom and for guiding me through this process.

TABLE OF CONTENTS

FIGURES

TABLES

ABBREVIATIONS

AAA	ATPases Associated with diverse cellular Activities
amc	*abstinence by mutual consent*
apm	*abnormal peroxisome morphology*
2,4-D	2,4-dichlorophenoxyacetic acid
2,4-DB	2,4-dichlorophenoxybutyric acid
cDNA	complementary DNA
Col-0	Columbia
DTT	dithiothreitol
EDTA	ethylenediaminetetraacetic acid
EMS	ethyl methane sulfonate
ER	endoplasmic reticulum
ERAD	endoplasmic reticulum associate degradation
F_1, F_2, etc.	filial generation 1, 2, etc.
FG	forward genetics
GFP	green fluorescent protein
IAA	indole-3-acetic acid
IBA	indole-3-butyric acid
L*er*	Landsberg *erecta*
M_1, M_2, etc.	mutagenized plant generation 1, 2, etc.
NAA	1-napthalacetic acid
PCR	polymerase chain reaction
ped	*peroxisome deficient*

PEX	peroxin
PMDH	peroxisomal malate dehydrogenase
PN	plant nutrient medium
PNS	plant nutrient medium with sucrose
PTS1/PTS2	peroxisome targeting signal type 1 and 2
RADAR	receptor accumulation and degradation in the absence of recycling
RG	reverse genetics
RING	really interesting new gene
RNA	ribonucleic acid
SC	synthetic complete
SH3	SRC homology 3 domain
T-DNA	transfer-DNA
T_1, T_2, etc.	transformant generation 1, 2, etc.
Tris	Tris hydroxymethylaminoethane
Triton	octylpheoxypolyethoxyethanol polyethylene glycol-p-isooctylphenyl ether
UBC	ubiquitin-conjugating enzyme
UTR	untranslated region
Ws	Wassilewskija
Wt	wild type
X-gal	5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside

Chapter 1: Introduction

1.1 Function of peroxisomes

Peroxisomes are small, membrane-bound organelles found in eukaryotic cells that compartmentalize many potentially hazardous reactions that are vital for cellular function. Fatty-acid β-oxidation and hydrogen peroxide degradation are common processes contained in all peroxisomes. However, additional specific reactions vary by species, developmental stage, and cell type. Plant peroxisomes have been implicated in essential processes, including germination, phytohormone biosynthesis, seedling establishment, seed oil quality, photosynthetic efficiency, and defense (reviewed in Hayashi and Nishimura, 2003).

In plants, peroxisomes are plastic and are able to modulate their contents in response to developmental and environmental cues (reviewed in Hayashi and Nishimura, 2006). In early seedling development, peroxisomes house enzymes required for the β-oxidation of fatty acids to acetyl-CoA and the glyoxylate cycle, which allows utilization of acetyl-CoA to provide energy prior to photosynthesis (reviewed in Beevers, 1993). Mature leaf peroxisomes are important for photorespiration and house enzymes necessary for conversion of Calvin-Benson cycle substrates (Somerville and Ogren, 1980; Somerville and Ogren, 1981). During certain cellular processes, such as when seedlings acquire photosynthetic capability and during senescence, peroxisome contents change to suit the cellular condition (reviewed in Johnson and Olsen, 2001).

1.2 Tools for studying physiological responses of peroxisomes in plants

Because plant peroxisomes are the sole site of fatty acid β-oxidation (Kindl, 1993) and the related process of IBA to IAA conversion (Zolman et al., 2000; Strader et al., 2010), sucrose dependence and IBA response can be used to assess peroxisomal function and in screens for mutants with reduced peroxisome activity.

1.2.A Fatty acid β-oxidation

Fatty acid β-oxidation is the process of breaking down lipids into acetyl-CoA for use as an energy source (Kindl, 1993). Oilseed plants, like Arabidopsis, rely on this process for seedling establishment until they are photosynthetically capable. Therefore, deficiencies in peroxisomal protein import that cause fatty acid β-oxidation deficiencies lead to an inability to convert stored energy to sucrose (Hayashi et al., 1998; Zolman et al., 2000). Sucrose dependence can be tested by screening for the ability of dark-grown seedlings to elongate their hypocotyls on medium with and without sucrose (e.g., Zolman et al., 2000). Furthermore, peroxisome defective mutants can be sucrose dependent even in the light, which can be measured by comparing root growth of seedlings grown with and without sucrose in the light (e.g., Adham et al., 2005).

1.2.B IBA β-oxidation

Auxins, such as IBA and IAA, are endogenous plant hormones that elicit many growth responses when applied exogenously, including inhibition of root elongation and promotion of lateral root formation (reviewed in Woodward and Bartel, 2005a). IBA and IAA differ by two carbon units in the carboxyl side chain. It has been suggested that IBA is converted to IAA by the removal of this two carbon unit in a manner similar to fatty

acid β-oxidation (Fawcett et al., 1960; Epstein and Lavee, 1984; Ludwig-Müller and Epstein, 1991). Evidence that this conversion is peroxisomally located is provided by the *IBA-response* (*ibr)* mutants, which are resistant to IBA but respond normally to IAA, and are defective in proteins required for peroxisomal biogenesis (Zolman et al., 2000; Zolman and Bartel, 2004; Woodward and Bartel, 2005b; Zolman et al., 2005; Ramón and Bartel, 2010), peroxisomal enzymes (Zolman et al., 2001a; Adham et al., 2005; Zolman et al., 2007; Zolman et al., 2008), or peroxisomal transporters (Zolman et al., 2001b). IBA resistance can be assayed under several conditions to evaluate primary root elongation and lateral root initiation.

Like IBA β-oxidation, the synthetic hormone 2,4-dichlorophenoxy acid (2,4-DB) can be metabolized to 2,4-dichorophenoxyacetic acid (2,4-D, Wain and Wightman, 1954). 2,4-DB resistance can be measured using the same assays as IBA resistance (Hayashi et al., 1998).

1.2.C Peroxisomal import assays

Peroxisomal matrix enzymes are directed to the peroxisome via peroxisome targeting signals (PTS) that are conserved across species. The two most commonly identified PTS are the PTS1, composed of Ser-Lys-Leu (SKL) or similar variants at the extreme carboxy-terminus of the protein (Gould et al., 1989; Mullen, 2002), and PTS2, composed of Arg-Leu-$(X)_5$-His-Leu near the amino-terminus of the protein (Osumi et al., 1991; Flynn et al., 1998). Because PTS2 signals are cleaved following peroxisomal import in mammals (Osumi et al., 1991; Swinkles et al., 1991) and plants (Preisig-Muller and Kindl, 1993; Kato et al., 1999), immunoblotting can be used to monitor PTS2 matrix protein import defects by analyzing the ratio of processed to unprocessed PTS2 protein.

PTS2 processing is catalyzed by a PTS1 protein (Schumann et al., 2008), and because both proteins must be properly localized for processing to occur, PTS2 processing can also be a measure of PTS1 import (Figure 1.1).

1.3 PEX proteins are necessary for peroxisome biogenesis

PTS-containing proteins are encoded by nuclear genes and are post-translationally imported through the action of the peroxin (PEX) proteins. In yeast and mammals, more than 30 peroxins have been identified (reviewed in Wanders and Waterham, 2004). Several approaches have been used to identify plant peroxins, including forward and reverse genetics and sequence homology studies (Table 1.1). Using these methods, PEX2 (Hu et al., 2002), PEX4 (Zolman et al., 2005), PEX5 (Zolman et al., 2000), PEX6 (Zolman and Bartel, 2004), PEX7 (Woodward and Bartel, 2005b), PEX10 (Schumann et al., 2003), PEX11 (Lingard et al., 2006; Orth et al., 2007), PEX12 (Fan et al., 2005; Mano et al., 2006), PEX13 (Mano et al., 2006; Boisson-Dernier et al., 2008), PEX14 (Hayashi et al., 2000), PEX16 (Lin et al., 1999), and PEX22 (Zolman et al., 2005) have been identified and preliminarily characterized in plants. Genetic studies have demonstrated the importance of these PEX proteins in embryogenesis, seedling establishment, photomorphogenesis, and photorespiration (reviewed in Hayashi and Nishimura, 2003)

As outlined in the following sections, peroxisome matrix import can be conceptually divided into four steps consisting of cargo recognition, docking to the membrane, cargo translocation and release, and receptor recycling (Figure 1.1).

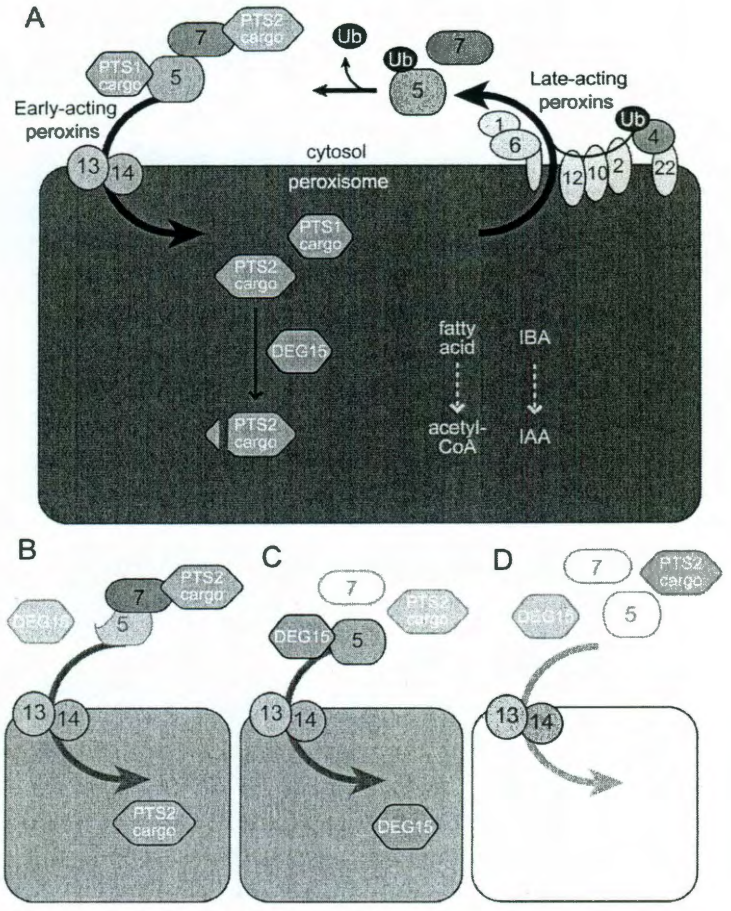

Figure 1.1 Peroxisomal import and receptor recycling is required for matrix enzyme import and processing.

A. Model from peroxisome matrix protein import in Arabidopsis based on studies in Arabidopsis, yeast, and mammals. In the cytosol, the receptors PEX5 and PEX7 bind PTS1 and PTS2-containing proteins, respectively. The receptor-cargo complex binds the docking complex composed of PEX13 and PEX14 at the peroxisomal membrane. Upon docking, the receptors release their cargo into the peroxisomal matrix. PEX5 is recycled using a mono-ubiquitin modification. Ubiquitination is performed by the RING finger complex composed of PEX2, PEX10, and PEX12 with the assistance of the ubiquitin conjugating enzyme, PEX4 and the membrane anchor PEX22. The AAA ATPases PEX1 and PEX6 are anchored to the peroxisomal membrane by PEX15/26 in yeast and mammals; the Arabidopsis anchor has not been identified. PEX1 and PEX6 recognize the departing PEX5 and are required for PEX5 release into the cytosol. PEX5 is deubiquitinated in the cytosol and free to undergo further rounds of import. PEX7 recycling is not well understood. Upon entrance into the peroxisomal matrix, PTS2 proteins are processed to mature isoforms via PTS2 cleavage. The protease responsible for PTS2 cleavage, DEG15, is itself a PTS1 enzyme. See text for references.

B-D. Depiction of possible defects that result in PTS2 processing defects. Illustration of defects resulting from impairing PTS1 import (B), PTS2 import (C), or both (D). For proper PTS2 processing, both PTS1 and PTS2 import pathways must be intac

Table 1.1 Summary of *Arabidopsis pex* mutants

Gene	Mutant[1]	Isolation[2]	Reference	Characteristics of mutants		
				Protein Level	Physiological Defects	Molecular Defects
PEX1/At5g08470	*pex1i*	RG (RNAi)	(Nito et al., 2007)	Unknown	2,4-DB resistance and sucrose dependence	Reduced PTS1 and PTS2 import
PEX2/At1g79810	*ted3*	FG	(Hu et al., 2002)	Unknown	Suppression of *det1*	None reported
	pex2	RG (T-DNA)	(Hu et al., 2002)	Unknown	Embryo lethality	None reported
	pex2i	RG (RNAi)	(Nito et al., 2007)	Unknown	2,4-DB resistance and sucrose dependence	Reduced PTS1 and PTS2 import
PEX3.1/At1g48640	*pex3.1/3.2i*	RG (RNAi)	(Nito et al., 2007)	Unknown	Slight sucrose dependence	None detected
PEX3.2/At3g18160	*pex3.1/3.2i*	RG (RNAi)	(Nito et al., 2007)	Unknown	Slight sucrose dependence	None detected
PEX4/At5g25760	***pex4-1***	FG	(Zolman et al., 2005)	Unknown	IBA resistance and sucrose dependence	Reduced PTS2 processing; normal PEX5 levels
PEX5/At5g56290	***pex5-1***	FG	(Zolman et al., 2000)	Unchanged	IBA resistance and sucrose dependence	Reduced PTS2 processing and PTS2 import; normal PTS1 import
	pex5-10	RG (T-DNA)	(Zolman et al., 2005)	Reduced	IBA resistance and sucrose dependence; delayed development	Reduced PTS2 processing
	pex5i	RG (RNAi)	(Hayashi et al., 2005)	Reduced	2,4-DB resistance and sucrose dependence	Reduced PTS1 and PTS2 import
PEX6/At1g03000	***pex6-1***	FG	(Zolman and Bartel, 2004)	Unchanged (this work)	IBA resistance and sucrose dependence; small, pale green adult plant	Reduced PTS2 processing; reduced PTS1 import (this work); reduced PEX5 levels
PEX7/At1g29260	***pex7-1***	RG (T-DNA)	(Woodward and Bartel, 2005)	Reduced	IBA resistance and sucrose dependence	Reduced PTS2 processing, PTS2 import
	pex7-2	FG	(Ramon and Bartel, 2010)	Reduced	IBA resistance and sucrose dependence	Reduced PTS2 processing, PTS1 and PTS2 import
	pex7i	RG (RNAi)	(Hayashi et al., 2005)	Reduced	2,4-DB resistance and sucrose dependence	Reduced PTS2 import; normal PTS1 import
PEX10/At2g26350	*pex10*	RG (T-DNA)	(Schumann et al., 2003; Sparkes et al., 2003)	Unknown	Embryo lethality	Unknown
PEX11.1/At1g47750	*PEX11a/bi*	RG (RNAi)	(Nito et al., 2007)	Unknown	None detected	None detected
PEX11.2/At3g47430	*PEX11a/bi*	RG (RNAi)	(Nito et al., 2007)	Unknown	None detected	None detected
PEX11.3/At1g01820	*PEX11c/d/ei*	RG (RNAi)	(Nito et al., 2007)	Unknown	Slight sucrose dependence	None detected
PEX11.4/At2g45740	*PEX11c/d/ei*	RG (RNAi)	(Nito et al., 2007)	Unknown	Slight sucrose dependence	None detected

Table 1.1 continued

Gene	Mutant[1]	Isolation[2]	Reference	Characteristics of mutants		
				Protein Level	Physiological Defects	Molecular Defects
PEX11.4/At2g45740	*PEX11c/d/ei*	RG (RNAi)	(Nito et al., 2007)	Unknown	Slight sucrose dependence	None detected
PEX11.5/At3g61070	*PEX11c/d/ei*	RG (RNAi)	(Nito et al., 2007)	Unknown	Slight sucrose dependence	None detected
PEX12/At3g04460	*apm4*	FG	(Mano et al., 2006)	Unknown	2,4-DB resistance and sucrose dependent Embryo lethality	PTS1 and PTS2 import defects; reduced PTS2 processing
	pex12	RG (T-DNA)	(Fan et al., 2005)	Unknown	IBA resistance and sucrose dependence	PTS1 import defect
	pex12i	RG (RNAi)	(Nito et al., 2007)	Unknown	2,4-DB resistance and sucrose dependence	Reduced PTS1 and PTS2 import
PEX13/At3g07560	***pex13-1***	RG (T-DNA)	This work	Unchanged (this work)	No dramatic defects (this work)	No dramatic defects (this work)
	pex13-4	FG	This work	Reduced (this work)	IBA resistance and sucrose dependence (this work)	Reduced PTS2 processing (this work)
	amc	FG	(Boisson-Dernier et al., 2008)	Unknown	Gametophyte lethality	Unknown
	apm2	FG	(Mano et al., 2006)	Reduced	No dramatic defects, slight 2,4-DB resistance and sucrose dependence	Reduced PTS1 and PTS2 import; reduced PTS2 processing
	pex13i	RG (RNAi)	(Nito et al., 2007)	Unknown	2,4-DB resistance and sucrose dependence	Reduced PTS1 and PTS2 import
PEX14/At5g62810	*ped2*	FG	(Hayashi et al., 2000)	Reduced	2,4-DB resistance and sucrose dependence	Mild PTS1 and PTS2 import defect; reduced PTS2 processing
	pex14-2	RG (T-DNA)	(Monroe-Augustus et al., 2010)	Reduced	IBA resistance and sucrose dependence; small adult plant	Reduced PTS2 processing, PTS1 and PTS2 import
PEX16/At2g45690	*sse1*	RG (T-DNA)	(Lin et al., 2004)	Unknown	Sucrose dependence	Unknown
	pex16i	RG (RNAi)	(Nito et al., 2007)	Unknown	2,4-DB resistance	None detected
PEX17/At4g18195 At4g18197 At4g18205	---					
PEX19.1/At3g03490	*pex19-1i*	RG (RNAi)	(Nito et al., 2007)	Unknown	None detected	None detected
PEX19.2/At5g17550	*pex19-2i*	RG (RNAi)	(Nito et al., 2007)	Unknown	None detected	None detected
PEX22/At3g21865	*pex22-1*	RG (T-DNA)	(Zolman et al., 2005)	Unknown	None detected	None detected

[1]Mutants in bold were used in this study and characteristics listed are from referenced source unless otherwise noted. [2]FG, forward genetics from EMS-mutagenized seeds; RG (RNAi), reverse genetics from RNAi lines; RG (T-DNA), reverse genetics from T-DNA insertion lines.

1.4 Cargo-receptor recognition and binding: PEX5 and PEX7

The first step in peroxisomal matrix protein import is receptor binding in the cytosol. The well characterized PTS1 and PTS2 peroxisomal targeting signals (Section 1.2.C) are recognized by the receptors PEX5 and PEX7, respectively (Figure 1.1A).

In yeast, PEX5 and PEX7 import peroxisomal matrix proteins independently of one another. The carboxy-terminal tetratricopeptide repeats of PEX5 are necessary for PTS1 recognition (Dodt et al., 1995; Terlecky et al., 1995; Gatto et al., 2000). PEX7 is a member of the WD-40 repeat family (Marzioch et al., 1994). In *Saccharomyces cerevisiae*, PEX7 requires the co-receptors PEX18 and PEX21 for PTS2 import (Dodt et al., 2001; Otera et al., 2002). In other fungi, such as *Pichia pastoris* and *Hansenula polymorpha*, PEX20 is utilized as a co-receptor in place of PEX18 and PEX21 (Sichting et al., 2003; Otzen et al., 2005).

In contrast to yeast, mammalian PTS2 import is dependent on direct PEX5-PEX7 interaction. Mammals have long (PEX5L) and short (PEX5S) isoforms of PEX5 that result from alternative splicing (Otera et al., 1998). PEX5S can bind and import PTS1 cargo independently of PEX7. PEX5L binds PTS1 cargo and also PEX7 (Matsumura et al., 2000; Otera et al., 2000), and PEX7-PTS2 import are dependent on PEX5L binding (Braverman et al., 1998).

Some plant species alternatively splice two isoforms of PEX5. For example, rice has both PEX5L and PEX5S isoforms. Similar to mammals, rice PEX5S does not bind PEX7 and PEX5L does bind PEX7 (Lee et al., 2006). In contrast to rice, the single Arabidopsis PEX5 isoform resembles PEX5L, interacts with PEX7, and is required for

both PTS1 and PTS2 import (Hayashi et al., 2005; Woodward and Bartel, 2005b; Ramón and Bartel, 2010).

In Arabidopsis, mutations in *PEX5* result in sucrose dependence, IBA resistance, and PTS2 processing defects (Zolman et al., 2000; Woodward and Bartel, 2005b; Ramón and Bartel, 2010). Two alleles of *pex5* have been characterized: *pex5-1*, a missense mutation (Zolman et al., 2000), and *pex5-10*, a mutant resulting from a T-DNA insertion in exon five (Zolman et al., 2005; Khan and Zolman, 2010). Interestingly, the *pex5-1* lesion is in the same region required for PEX5 binding to PEX7 in mammals, and Arabidopsis *pex5-1* exhibits PTS2 import defects, suggesting that PEX7-mediated PTS2 import is dependent on PEX5 binding (Woodward and Bartel, 2005b). In contrast, *pex5-1* imports PTS1 proteins normally (Woodward and Bartel, 2005b). In the Arabidopsis *pex5-10* T-DNA allele, full length PEX5 is undetectable, but a truncated pex5-10 protein can be detected (Zolman et al., 2005). Because PEX5 is the PTS1 receptor, and because PEX7 is dependent on PEX5, both PTS1 and PTS2 import are defective in *pex5-10* (Ramón and Bartel, 2010).

Two *pex7* mutants have been characterized in Arabidopsis. The *pex7-1* allele results from a T-DNA insertion 95 bp upstream of the start codon in the *PEX7* 5' UTR (Woodward and Bartel, 2005b). The *pex7-2* missense mutation causes an amino acid change in the second WD-40 domain (Ramón and Bartel, 2010). Both mutants are IBA resistant and are defective in PTS2 processing and peroxisomal import (Woodward and Bartel, 2005b; Ramón and Bartel, 2010). Additionally, when grown in the light, both *pex7* mutants fail to accumulate not only PEX7, but also PEX5 (Ramón and Bartel,

2010). Because a *pex7* mutation can disrupt PEX5 stability, PEX5 is likely dependent on PEX7 for import, recycling, or stability in the cytosol.

1.5 Docking to the peroxisomal membrane: PEX13 and PEX14

After the cargo has been secured by the receptors, the cargo-receptor complex binds the docking complex on the peroxisome membrane (Figure 1.1). The docking complex includes the peroxisomal membrane proteins PEX13 and PEX14 (reviewed in Lanyon-Hogg et al., 2010).

In yeast, PEX13 binds both PEX5 and PEX14 via a carboxy-terminal SH3 domain and PEX7 via an amino-terminal domain (Elgersma and Tabak, 1996; Erdmann and Blobel, 1996; Gould et al., 1996). The membrane peroxin PEX14 binds itself (Albertini et al., 1997), binds PEX5 through both the amino- and carboxy-terminal domains, and binds PEX7 via a carboxy-terminal domain (Albertini et al., 1997; Niederhoff et al., 2005; Williams et al., 2005). Furthermore, yeast PEX14 binds PTS2-cargo in a PEX7-dependent manner (Stein et al., 2002). PEX14 may serve as the initial receptor docking site because PEX5 has a higher affinity for PEX14 compared to PEX13 and this preference is enhanced when PEX5 is cargo-bound (Urquhart et al., 2000). Although PEX13 is needed to localize PEX14 to the peroxisome (Girzalsky et al., 1999), only a portion of PEX13 co-fractionates with PEX14 in yeast (Agne et al., 2003), suggesting that PEX13 may have other functions in addition to receptor docking or that the PEX13-PEX14 association is transient.

In mammals, PEX7 receptor-docking complex interactions are dependent on PEX5 because PEX5 bridges the binding of PEX7 to PEX14 (Otera et al., 2000). This

dependence of PEX7 on PEX5 in membrane docking is reflected in PTS2 import defects of mammalian *pex5* mutants (Braverman et al., 1998).

In Arabidopsis, both PEX14 (Hayashi et al., 2000) and PEX13 (Mano et al., 2006) are membrane associated. As in other systems, Arabidopsis PEX13 binds to PEX7 via an amino-terminal region (Mano et al., 2006) and PEX14 binds to PEX5 (Nito et al., 2002). However, Arabidopsis PEX13-PEX5, PEX13-PEX14, and PEX14-PEX7 interactions have not been reported.

Two Arabidopsis *pex13* alleles have been previously described. A GFP-PTS1-based screen for aberrant peroxisome morphology resulted in isolation of a weak *pex13* allele, *apm2* (Mano et al., 2006). *apm2* confers mild physiological phenotypes such as slight sucrose dependence and 2,4-DB resistance and results in a minor PTS1 import defect. In contrast, a null T-DNA allele, *abstinence by mutual consent* (*amc*), confers gametophyte lethality and cannot be propagated as a homozygote (Boisson-Dernier et al., 2008). In Chapter 3, I describe two new *pex13* alleles. *pex13-1* is a T-DNA mutant that lacks dramatic phenotypes independently but enhances or suppresses other *pex* mutants (Chapter 4 and 5). *pex13-4* is a missense mutation that displays strong IBA resistance, sucrose dependence, and PTS2 processing defects.

Until recently only one Arabidopsis *pex14* mutant had been described, *peroxisome defective2* (ped2, Hayashi et al., 2000). However, this mutant was isolated in the Landsberg accession unlike other *pex* mutants that were isolated in the Columbia accession. Because of the difference in background, direct comparisons cannot be made to existing *pex* mutants. The *ped2* lesion is a nonsense mutation midway through the coding sequence that results in small plants with partial PTS1 and transient PTS2 import

defects (Hayashi et al., 2000). Recently, an allelic series of *pex14* mutants were isolated and characterized in the Colombia accession (Monroe-Augustus et al., 2010). One of these alleles, *pex14-2*, has no detectable *PEX14* mRNA or PEX14 protein accumulation (Monroe-Augustus et al., 2010). *pex14-2* is IBA resistant and sucrose dependent, but has only partial PTS2 processing defects (Monroe-Augustus et al., 2010). The moderate phenotypes in *pex14-2* are in contrast to the leathality conferred by the *pex13* null allele, *amc*, and suggest that Arabidopsis PEX14 may have a minor or redundant role in peroxisome biogenesis (Monroe-Augustus et al., 2010).

1.6 Cargo translocation and release

Once bound at the peroxisome using the docking complex, matrix proteins have been suggested to traverse a pore in the peroxisomal membrane. Because pores of sufficient size to transport proteins have not been detected when peroxisome membranes are imaged, it has been suggested that these pores are dynamic or transient (McNew and Goodman, 1996; Meinecke et al., 2010).

In yeast, the pore in the peroxisomal membrane appears to be comprised of PEX5 oligomers (Meinecke et al., 2010). When isolated membrane complexes are incubated with cargo-loaded PEX5, a peroxisomal channel is activated (Meinecke et al., 2010). Furthermore, the channel can expand up to 9.25 nm (Meinecke et al., 2010), consistent with the ability of peroxisomes to import fully-folded, oligomeric proteins (McNew and Goodman, 1994).

In mammals and plants, isolated peroxisomal membranes have large-conductance channels when tested using patch-clamp techniques (Labarca et al., 1986; Lemmens et al., 1989; Reumann et al., 1995; Reumann et al., 1997; Reumann et al., 1998). In rat

liver, these channels are permeable to small solutes and water-soluble metabolites but not permeable to hydrophilic compounds such as NAD/H or CoA and its acetyl derivatives (Antonenkov et al., 2005). However, the protein constituents that make up these pores have not been described in either mammals or plants.

1.7 Ubiquitination of PEX5 and removal from the peroxisome: the "late-acting" peroxins

Following translocation of the cargo matrix enzyme, PEX5 and PEX7 are recycled to the cytosol for further rounds of import. Two processes for removal of PEX5 from the peroxisomal membrane have been proposed, and both mechanisms rely on ubiquitination of the PEX5 receptor (Figure 1.3, reviewed in Thoms and Erdmann, 2006). In contrast, little is known regarding the mechanisms of PEX7 recycling.

1.7.A PEX5 mono-ubiquitination and recycling

Mono-ubiquitination can regulate protein-protein interactions, alter protein function, and affect protein cellular localization (reviewed in Schnell and Hicke, 2003). Mono-ubiquitin modification is thought to be the marker for PEX5 retrotranslocation. Following extraction from the peroxisomal membrane, PEX5 is deubiquitinated for further rounds of import (reviewed in Thoms and Erdmann, 2006).

In yeast, PEX5 mono-ubiquitination occurs on a conserved Cys residue near the amino-terminus (Kragt et al., 2005; Williams et al., 2007). PEX5 mono-ubiquitination is mediated by the ubiquitin-protein ligase RING-finger peroxin PEX12 (Platta et al., 2009), which is found in complex with the RING-finger peroxins PEX2 and PEX10.

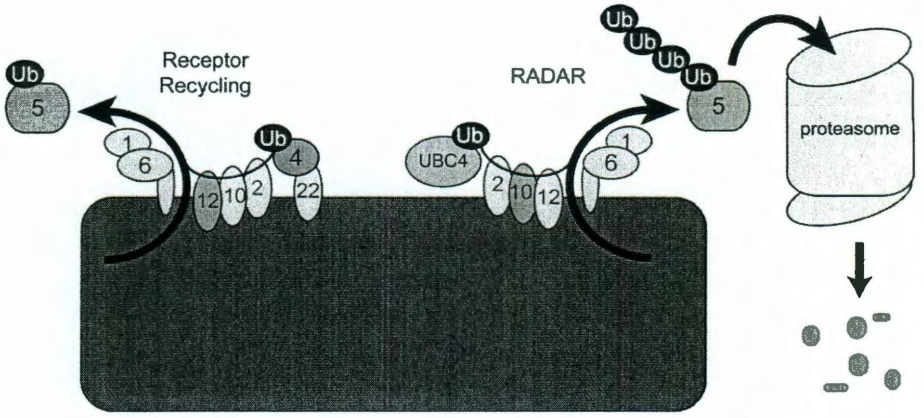

Figure 1.2 PEX5 recycling and degradation by RADAR.
In yeast, PEX5 recycling is dependent on the activity of the RING finger peroxin PEX12 and the UBC PEX4. Mono-ubiquitination is a signal to the AAA ATPase PEX1 and PEX6 to remove PEX5 from the peroxisome and release PEX5 into the cytosol. When recycling is defective, UBC4 and PEX10 poly-ubiquitinate PEX5. This modification results in PEX5 degradation by the proteasome. See text for references.

Mono-ubiquitination requires the activity of the ubiquitin-conjugating enzyme PEX4 (Collins et al., 2000) which is anchored to the peroxisomal membrane by PEX22 (Koller et al., 1999; Collins et al., 2000).

Following mono-ubiquitination, tagged PEX5 is recognized by an AAA ATPase complex composed of PEX6 and PEX1 and is removed from the peroxisome in an ATP-dependent process (Collins et al., 2000; Platta et al., 2005). PEX6 and PEX1 contain two conserved ATP-binding domains (Birschmann et al., 2005). PEX1-PEX6 interaction is ATP dependent (Faber et al., 1998). In yeast, mutations that disrupt ATP binding or hydrolysis also reduce ability to grow on oleate, a fatty-acid carbon source (Birschmann et al., 2005). The PEX1-PEX6 complex is anchored to the peroxisomal membrane by the peroxisomal membrane protein PEX15 (Birschmann et al., 2003).

In mammals, a PEX4 homolog has not been identified, but the cytosolic UBC5 is required for PEX5 mono-ubiquitinatation at the amnio-terminal Cys^{11} residue (Grou et al., 2008). Additionally, ubiquitin modification is required for PEX5 removal from the peroxisomal membrane in cell-free systems (Carvalho et al., 2007). Similar to yeast, mammalian PEX1 and PEX6 form a heteroligomer anchored to the peroxisomal membrane by PEX26 (Tamura et al., 2006). Disruption of the PEX1-PEX6-PEX26 complex is the most common cause of human peroxisomal biogenesis disorders (Geisbrecht et al., 1998).

In plants, ubiquitination of PEX5 has not been demonstrated, but genetic evidence suggests that Arabidopsis PEX5 is recycled in a manner similar to yeast PEX5 (Zolman and Bartel, 2004; Zolman et al., 2005). Additionally, the cysteine residue required for

PEX5 monoubiquitination in yeast and mammals is conserved in Arabidopsis PEX5, suggesting a conserved recycling mechanism across species (Williams et al., 2007).

In Arabidopsis, three *pex* mutants defective in late-acting peroxins have been characterized: *pex4-1*, *pex22-1*, and *pex6-1*. *pex4-1* was isolated in a screen for IBA resistance (Zolman et al., 2005) and is a missense allele resulting from Pro^{123} to Leu. The *pex4-1* mutation may interrupt PEX4-PEX22 interaction, as the lesion is located in the center of the region required for interaction of *Pichia* PEX22 and PEX4 (Koller et al., 1999). *pex4-1* has severe *pex* phenotypes, including IBA resistance and sucrose dependence (Zolman et al., 2005). However, PTS-tagged GFP is largely peroxisomal and PTS2 processing defects are slight in *pex4-1*, which seems inconsistent with the dramatic IBA resistance and sucrose dependence of the mutant (Zolman et al., 2005). In Chapter 8, I investigate this paradox and show that the phenotypes of *pex4-1* might result from PEX5 accumulation in the peroxisome rather than defective matrix protein import.

In Arabidopsis, PEX22 is a single-copy gene that does not display high similarity with yeast Pex22p and was identified in a PEX4-baited yeast two-hybrid screen (Zolman et al., 2005). *pex22-1* is a T-DNA mutant 107 base pairs upstream of the *PEX22* start codon that enhances the *pex4-1* phenotypes, although it lacks typical peroxisome-defective phenotypes independently (Zolman et al., 2005). Arabidopsis PEX4 and PEX22, when co-expressed, can partially rescue *S. cerevisiae pex4* and *pex22* mutant defects (Zolman et al., 2005). These results suggest, that as in yeast, Arabidopsis PEX4 and PEX22 act in a complex.

Arabidopsis *pex6-1* was also identified from a screen for mutants defective in IBA inhibition of primary root elongation (Zolman and Bartel, 2004). The *pex6-1* missense

mutation is an Arg^{766} to Gln and lies in the second AAA ATPase domain. In addition to resistance to IBA, *pex6-1* is sucrose dependent and has a PTS2 processing defect (Zolman and Bartel, 2004). However, like *pex4-1*, the severe physiological defects of *pex6-1* are inconsistent with the mild PTS2 processing defect.

1.7.B PEX5 poly-ubiquitination and RADAR

Dysfunctional PEX5 recycling can lead to reduced PEX5 levels through a process known as receptor accumulation and degradation in the absence of recycling (RADAR), a process similar to the ER quality control mechanism, endoplasmic reticulum-associated protein degradation (ERAD). In RADAR, when PEX5 is not removed via mono-ubiquitination and recycling, PEX5 can be poly-ubiquitinated and degraded by the proteasome (reviewed in Thoms and Erdmann, 2006).

In yeast, polyubiquitination of PEX5 is catalyzed by Ubc4 and the RING finger peroxin PEX10 (Williams et al., 2008) and polyubiquitinated PEX5 is only observed in deletion strains of the late-acting mutants *pex4*, *pex22*, *pex1*, *pex6*, and *pex15* (Platta et al., 2004; Kiel et al., 2005a; Kragt et al., 2005). Polyubiquitin chains have been identified on two distinct lysine residues in the amino-terminal end of PEX5 (Williams et al., 2007). Interestingly, the *Pichia* PEX7 co-receptor, PEX18/20, can also be polyubiquitinated at a similar Lys position (Leon et al., 2006). When the necessary lysine residues are mutated and polyubiqutination is blocked, PEX5 and PEX20 accumulate at the peroxisome (Leon et al., 2006).

In plants, yeast, and mammals, *pex6* mutants have reduced PEX5 levels (Dodt and Gould, 1996; Collins et al., 2000; Zolman and Bartel, 2004), consistent with the RADAR pathway model. Moreover, overexpressing PEX5 in Arabidopsis *pex6-1* plants partially

rescues many, but not all, of *pex6-1* defects including the general growth defects and sucrose dependence, indicating that reduced PEX5 contributes to *pex6-1* phenotypes (Zolman and Bartel, 2004).

There are discrepancies in PEX5 levels in *pex4* mutants across species. *Pichia pastoris* and *Hansenula polymorpha pex4* mutants have reduced PEX5 levels (Koller et al., 1999; Collins et al., 2000; Kiel et al., 2005b), and PEX5 accumulates in peroxisomes in *Pichia pastoris pex4* mutants (Collins et al., 2000). However, *Arabidopsis* (Zolman et al., 2005) and *S. cerevisiae* (Platta et al., 2004; Kiel et al., 2005a) *pex4* mutants have normal PEX5 levels.

1.8 In this thesis

In this thesis, I investigate the role of balance in peroxisomal receptor import and recycling using a series of *pex* mutants. In Chapter 3, I characterize two new *pex13* alleles. To explore the role of balance in peroxisomal import and recycling, I characterize physiological and molecular phenotypes that result when *pex13-1* is combined with early-acting mutants *pex5-1*, *pex5-10*, and *pex14-2* (Chapter 4) and with late-acting mutants *pex4-1* and *pex6-1* (Chapter 5). Some of the results in Chapters 3-5 have been published (Ratzel et al., 2010). In Chapter 6, I characterize *pex7* alleles using *pex13* double mutant analysis established in earlier chapters. Lastly, in Chapter 7, I investigate the molecular and physiological consequences of PEX5 accumulation in the late-acting mutants *pex4-1* and *pex6-1*.

In the appendix, I describe the mutant screens where I identified, characterized, and mapped *pex4-1* suppressors (Appendix A) and *pex6-1* suppressors (Appendix B). I also include the papers that resulted from supporting work I performed in characterizing a

pex14 allelic series (Appendix C) and characterization of *ecr1-1*, a mutant in auxin response (Appendix D).

Chapter 2: Materials and Methods

2.1 Plant material

All mutants were in the *Arabidopsis thaliana* Col-0 accession. The *pex13-1* (SALK_006744) and *pex14-2* (SALK_007441) mutants were from the Salk Institute T-DNA insertion collection (Alonso et al., 2003). The *pex13-4* mutant was identified by Marta Bjornson and Andrew Woodward from an EMS mutagenesis screen for sucrose dependent, IBA resistant seedlings. *pex13-1*, *pex13-4*, and *pex14-2* were backcrossed once prior to analysis. The *pex13-1* genotype was determined by PCR amplification of wild-type *PEX13* with PEX13-1 (5′-AGAATTCAATAAATCGAGACCCTAAAAT-3′) and PEX13-2 (5′-TATAGGGGCTGATACATAATAACCTAAAA-3′) which yielded a 287-bp product, and amplification across the *pex13-1* T-DNA junction with PEX13-1 and LB1-SALK (Alonso et al., 2003), which yielded a 300-bp product. The PEX13-1 plus LB1-SALK amplicon was sequenced to determine the position of the *pex13-1* T-DNA insertion. The *pex13-4* genotype was determined by PCR amplification with PEX13-17 (5′-CCATTTCATTGTTCTCATGTTAACCAATC-3′) and PEX13-18dCAPSEcoRI (5′-CAAGCATACGCAGTCAAATCTTGCGAATT-3′) which yielded a 111-bp product. Subsequent restriction digest with EcoRI resulted in 106-bp fragment for wild-type *PEX13*, whereas the *pex13-4* fragment is not digested. The genotypes of *pex4-1* (Zolman et al., 2005), *pex5-1* (Zolman et al., 2000), *pex5-10* (Woodward and Bartel, 2005b), *pex6-1* (Zolman and Bartel, 2004), *pex7-1* (Woodward and Bartel, 2005b), *pex7-2* (Ramón and Bartel, 2010), and *pex14-2* (Monroe-Augustus et al., 2010) were assessed using PCR-based amplification as described previously.

2.2 Arabidopsis growth conditions

Seeds were surface-sterilized, stratified overnight at 4°C, and plated on plant nutrient medium (PN, Haughn and Somerville, 1986) solidified with 0.6% [w/v] agar and supplemented with 0.5% [w/v] sucrose (PNS) or ethanol-dissolved hormone stock solutions as indicated. After 1-2 weeks, plants were moved to soil and grown at 22°C under continuous white light.

2.3 Phenotypic analysis

Seedlings were grown at 22°C for 1 day under continuous white light followed by 4 days in darkness (sucrose dependence in the dark assays) or continuous yellow-filtered (Stasinopoulos and Hangarter, 1990) light for 8 days (IBA resistance and sucrose dependence in the light assays). For lateral root assays, seedlings were grown under yellow-filtered light on PNS for 4 days followed by 4 days on PNS containing 10 μM IBA or the equivalent amount of ethanol.

2.4 Genetic analysis

2.4.A Plant DNA isolation

DNA was isolated from plant tissue as described (Celenza et al., 1995). In summary, plant tissue was harvested and frozen on dry ice. Frozen tissue was ground with a pestle and 10 μL of 0.5 N NaOH was added. The sample was heated at 100°C for 30 seconds and 100 μL of neutralization buffer (0.2 M Tris pH 8.0, 1 mM EDTA) was added. 2 μL of the resulting extract was used as template DNA in 30 μL PCR reactions.

2.4.B Plant cDNA preparation

cDNA was prepared from 5 μg wild-type Arabidopsis (Col-0) RNA (Section 2.13). First-strand cDNAs were synthesized using the SuperScript III First-Strand Synthesis System standard protocol using random hexamer priming and SuperScript III Reverse Transcriptase (Invitrogen, Carlsbad, CA). cDNAs of interest were amplified by PCR from the first-strand reaction using gene-specific primers.

2.4.C Construct preparation

Inserts for constructs constructs used in this study were amplified by PCR using ExTaq (TaKaRa Bio Inc.) with a *SalI* site 5′ of the ATG to allow in-frame cloning in the pBI770 and pBI771 yeast two-hybrid vectors (Kohalmi et al., 1998) and a *NotI* site immediately downstream of the stop codon. cDNAs of interest were amplified from cDNA prepared from wild-type Arabidopsis mRNA (Section 2.4.B). Specific primers used for each construct are listed in Table 2.1. Amplicons were cloned into pCR4-TOPO (Invitrogen, Carlsbad, CA) before subcloning into various vectors. Primers used and constructs generated are summarized in Table 2.1.

2.4.C.1 Overexpression constructs

To generate overexpression constructs, the PEX13 or PEX14 coding sequence was subcloned as a SalI/NotI fragment from pCR4-PEX13 or pCR4-PEX14, respectively, into XhoI/NotI-cut 35SpBARN (LeClere and Bartel, 2001) to give 35S-PEX13 or 35S-PEX14, in which the cDNA is driven by the strong 35S promoter.

2.4.C.2 Yeast two-hybrid constructs

Inserts for the yeast two-hybrid constructs were fused to the Gal4-DNA-binding domain using the pBI770 vector (Kohalmi et al., 1998) or the Gal4-activation domain using the pBI771 vector (Kohalmi et al., 1998). To generate PEX13 and PEX14 yeast-two hybrid constructs, the PEX13, truncated PEX13, or PEX14 coding sequence was subcloned as a SalI/NotI fragment from pCR4-TOPO into SalI/NotI-cut pBI770 or pBI771 (Kohalmi et al., 1998) to give pBI770-PEX13 or pBI771-PEX13 and the various combinations (described in Chapter 8). To generate pBI770-PEX6, the coding sequence was subcloned as a SalI/NotI fragment from pCR4-TOPO into SalI/NotI-cut pBI770 (Kohalmi et al., 1998) to give pBI770-PEX6. Inserts were confirmed by sequencing.

2.4.C.3 TAP-tag constructs

The PEX6 insert was fused to the TAP-tag using pEG205 (Earley et al., 2006). The PEX6 coding sequence lacking the stop codon was amplified and cloned into pENTR-TOPO (Invitrogen, Carlsbad, CA). The entry vector carrying PEX6 was digested with MluI, gel purified, and the insert was transfered into pEG205 (Earley et al., 2006) using the standard reaction for the Gateway LR reaction to produce pEG206-PEX6.

Table 2.1 Primers for plasmid constructs used in this study

cDNA	Primer Name	Primer Sequence
PEX13	PEX13-SalI	GTCGACGA**ATG**GCGTCTCAGCCTGCAGG
	PEX13-NotI	GCGGCCGC**TTA**GTTGCCCCATACATTG
PEX13 (1-100)	PEX13-SalI	GTCGACGA**ATG**GCGTCTCAGCCTGCAGG
	PEX13(t)-NotI	GCGGCCGCT**TTA**TGACCCATACCAGAGGTCATA
PEX13 (213-305)	SalI-PEX13t2e(213)	TAGTCGACGAGCG**ATG**CTTATAG
	PEX13-NotI	GCGGCCGC**TTA**GTTGCCCCATACATTG
PEX14	PEX14-SalI	GTCGACGA**ATG**GCAACTCATCAGCAAA
	PEX14-NotI	GCGGCCGC**TTA**GTTCCCTTCCTGGCTGATAT
PEX6	fENTR-PEX6	CACC**ATG**GTGGAGAGACGGAATCCTCTGG
	rENTR-PEX6	GCTCGAACGGCCTTGAAATTGATCTCGA

Underlined nucleotides indicate introduced restriction sites; start and stop codons are in bold.

Table 2.2 Bartel lab stock numbers for plasmids used in this study

	Vector					
	TOPO	pENTR-TOPO	pBI770	pBI771	35SpBARN	
Insert	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*E. coli*	*A. tumefacians*
PEX13	2849		2850	2851	2852	2853
PEX13 (1-100)	2854		2855	2856		
PEX13 (213-304)	2857		2858	2859		
PEX14	2860		2861	2862	2863	2864
PEX6		2865				

2.5 *E. coli* transformation and growth conditions

TOP10 (Invitrogen) and NEB5-α (New England Biolabs) chemically competent cells were used in this study. For transformations, cells were thawed on ice for 10 minutes, 1-5 μL of DNA was added, and the cells were left on ice for an additional 30 minutes. Heat shock was performed at 42°C for 30 seconds and the mixture was placed on ice for 5 minutes. The cells were allowed to recover in 250 μL SOC (Ausubel et al., 1995) for one hour at 37°C. Transformations were plated on Luria Broth (LB) plates (Ausubel et al., 1995) with selective antibiotics and grown at 37°C overnight.

2.6 *Agrobacterium tumefacions* transformations and growth conditions

Agrobacterium tumefacians strain GV3101 (Koncz et al., 1992) was transformed using electroporation (Ausubel et al., 1995) and transformants were selected on LB plates supplemented with 50 μg/mL kanamycin and 50 μg/mL gentamycin. Transformation plates were incubated at room temperature for 4-5 days.

Plant lines were transformed with GV3101 strains containing plasmids of interest using the floral dip method (Clough and Bent, 1998). Transformed GV3101 strains were grown in 600 mL of LB supplemented with 50 μg/mL kanamycin and 50 μg/mL gentamycin for two days at 30°C. Cells were harvested by centrifugation and resuspended in 500 mL infiltration medium (Clough and Bent, 1998).

2.7 Arabidopsis transformation and growth conditions

Plants for transformation were moved to soil and grown at 22°C under continuous white light and primary inflorescences were removed to promote secondary growth. Plants were dipped in *Agrobacterium* resuspended in infiltration medium (Section 2.6),

covered with plastic wrap, and incubated in the dark overnight. The following day, plants were rinsed with water and placed under continuous white light. Floral dip was repeated for increased transformation efficiency.

Transformants were isolated in the T_1 generation by selecting seedlings that were BASTA resistant following growth on PNS supplemented with 7.5 μg/mL BASTA and 20 μg/mL Timentin. T_2 seeds were grown on PNS supplemented with 7.5 μg/mL BASTA and lines segregating 75% BASTA resistance were moved to soil. Preliminary analysis was carried out in T_2 lines and homozygous T_3 lines were used for final analysis.

2.8 Yeast transformation and growth conditions

Saccharomyces cervesiae YPB2 (*MAT***a**, *ura3-52 his3-200 ade2-101 lys2-801 trp1-901 leu2-3,112 can*^{R} *gal4-542 gal80-338 LYS2::GALI*$_{UAS}$*-LEU2*$_{TATA}$*-HIS3 URA3.:.GAL4-MEL1*$_{UAS}$*-MEL1*$_{TATA}$*- lacZ*) was grown in YPD (Ausubel et al., 1995) medium overnight. For each transformation (Gietz and Schiestl, 1995), cells from 0.5 mL overnight culture were harvested by centrifugation for 30 seconds and resuspended in 50 μL sterile water. 10 μL single-stranded carrier DNA (10 mg/mL salmon sperm DNA), 4 μL plasmid DNA, and 0.5 mL yeast transformation buffer (0.1 M lithium acetate, 10 mM Tris pH 7.5, 1 mM EDTA, 0.1 M DTT, 40% [w/v] polyethylene glycol 3350) was added and the mixture was vortexed thoroughly and incubated overnight at room temperature. Following incubation, the cells were harvested by centrifugation, resuspended in 0.5 mL sterile water, and plated on synthetic complete medium lacking leucine and tryptophan (SC-Leu-Trp).

2.9 Yeast two-hybrid analysis

Yeast two-hybrid analysis was performed in YPB2. Transformants were grown on synthetic complete (SC) media without Leu or Trp (SC-Leu-Trp). Two assays were used to test for interaction: growth on medium lacking His and a colony filter-lift β-galactosidase assay. For the growth assay, single colonies were grown in liquid culture and plated on SC-Leu-Trp with His or without His and supplemented with 2 mM 3-aminotriazole where growth on medium lacking His indicated interaction. For the β-gal assay, lines were grown on SC-Leu-Trp plates, colonies were transferred to nylon membranes (Scheicher and Schuell, Keene, NH) via filter lift, frozen in liquid nitrogen for 30 seconds, and incubated with Z Buffer (60 mM Na_2HPO_4, 40 mM $NaH_2PO_4H_2O$, 10 mM KCl, 1 mM $MgSO_4$, 39 mM 2-mercaptoethanol, pH 7.0) supplemented with 1 mg/mL 5-bromo-4-chloro-3-indolyl-ß-D-galactopyranoside (X-gal). Development of blue color indicated interaction.

2.10 Double mutant generation and isolation

Double mutants were generated by crossing. F_1 seeds were surface sterilized and grown on PNS plates for 8-16 days at 22°C under continuous white light before being transferred to soil and grown at 22°C under continuous white light. F_2 seeds were harvested, plated on PNS, and DNA was isolated from a leaf (Section 2.4.A) after 5-12 days. PCR-based markers were used for genotype determination (Section 2.1) and plants of interest were moved to soil. The *pex5-1 pex13-1*, *pex14-2 pex13-1*, *pex4-1 pex13-1*, *pex6-1 pex13-1*, and *pex7-1 pex13-1* double mutants were isolated by Matthew Lingard.

2.11 Western blot analysis

Protein was extracted from 8 seedlings grown on PNS for 5 days under continuous white light or 1 day light followed by 4 days of darkness by grinding frozen tissue and adding 15 μL NuPAGE 2x loading buffer (Invitrogen, Carlsbad, CA). After centrifugation, the supernatant was transferred to a fresh tube with 1.6 μL 0.5 M DTT and boiled at 100°C for 5 minutes. Samples were loaded onto NuPAGE 10% Bis-Tris gels (Invitrogen) next to prestained protein markers (P7708S, New England Biolabs, Beverly, MA) and Cruz Markers (Santa Cruz Biotechnology, Santa Cruz, CA). After electrophoresis, proteins were transferred for 35 minutes at 24 V to a Hybond ECL nitrocellulose membrane (Amersham Pharmacia Biotech, Piscataway, NJ) using NuPAGE transfer buffer (Invitrogen). Membranes were dried at 65°C for 15 minutes, rehydrated in Tris-buffered saline with 0.1% [v/v] Tween-20 (TBST, Ausubel et al., 1995), blocked at 4°C with 8% [w/v] non-fat dry milk in TBST, and incubated overnight at 4°C with the following primary antibodies diluted in 8% non-fat milk in TBST: rabbit anti-PEX5 (1:100 dilution, Zolman and Bartel, 2004), rabbit anti-PEX6 (1:10,000 dilution; prepared from rabbits inoculated with a recombinant protein including the first 350 amino acids of PEX6/At1g03000), rabbit anti-PEX7 (1:2500 dilution, Ramón and Bartel, 2010), rabbit anti-PEX13 (1:500 dilution, Mano et al., 2006), rabbit anti-PEX14 (1:10,000 dilution, Lingard and Bartel, 2009), rabbit anti-PED1 (1:2000 dilution, Lingard et al., 2009), rabbit anti-PMDH2 (1:2000 dilution, Pracharoenwattana et al., 2007), rabbit anti-MLS (1:25000 dilution, Olsen et al., 1993), rabbit anti-catalase (1:4000 dilution, Kunce et al., 1988), mouse anti-complex V subunit α (1:2000 dilution, MS507, MitoScience, Eugene, OR), or mouse anti-HSC70 (1:500 dilution, SPA-817, StressGen

Biotechnologies). Horseradish peroxidase-linked goat anti-rabbit or anti-mouse IgG (SC-2030 or SC-2031, Santa Cruz Biotechnology, Santa Cruz, CA) were used as secondary antibodies and were visualized using LumiGLO reagent (Cell Signaling Technology, Danvers, MA).

2.12 Cell fractionation

500 mg of 5-day-old light-grown seedlings were roughly chopped with a razor blade in 1 mL ice-cold fractionation buffer (150 mM Tris pH 7.6, 10 mM KCl, 1 mM EDTA, 1 mM DTT, 100 mM sucrose, 1 x protease inhibitor cocktail [P9559, Sigma]) followed by homogenization in a dounce homogenizer on ice. The homogenate was filtered through Miracloth (Calbiochem, La Jolla, CA), and 50 µL were removed as the "homogenate" (H) fraction. The remaining homogenate was centrifuged at 690 rpm at 4°C to remove cellular debris. The supernatant was centrifuged at 12,000 rpm and removed to give the "soluble" (S) fraction, and the pellet was washed once with fractionation buffer and resuspended in 40 µL of fractionation buffer to give the "pellet" (P) fraction. Following fractionation, 10 µL of the H, S, and P fractions were mixed with 10 µL of NuPAGE 2x loading buffer (Invitrogen, Carlsbad, CA) and processed for immunoblot analysis as described in Section 2.11.

2.13 RNA analysis

Seedlings were grown in white light on filter paper-covered PNS for 10 days. RNA was extracted with the RNeasy kit (Qiagen, Valencia, CA) using the standard extraction protocol. Total RNA was subjected to RNA gel blot analysis as previously described (Dugas and Bartel, 2008). Digoxigenin-labeled probes were amplified using a

PCR DIG Probe Synthesis Kit (Table 2.2, Roche, Indianapolis, IN) according to the manufacturer's instructions.

Table 2.3 Primers used to generate DIG probes for RNA gel blot analysis

mRNA	Primer Name	Primer Sequence
PEX13	PEX13-6	CTAGACCTTGGGAACAGCAGAAT
	PEX13-14	CTGCTGCATCTTTCTAGGCCTTGTTCTCAC
PEX14	PEX14-1	AGAGGCTACTAAGCCTGCTAATGA
	PEX14-2	ATGTTGCTGTTCTGTTTCTTCTTG

Chapter 3: Characterization of Arabidopsis *pex13* Mutants*

PEX13 is a component of the docking complex and is important for peroxisomal matrix import. Two Arabidopsis *pex13* alleles have been previously characterized. A GFP-PTS1-based screen for mutants with aberrant peroxisome morphology identified the weak *pex13* allele *apm2* (Mano et al., 2006). *apm2* has only slight PTS1 import defects and no PTS2 import defect (Mano et al., 2006). Consistent with a mild import defect, *apm2* is only slightly sucrose dependent or 2,4-DB resistant (Mano et al., 2006). A second *pex13* allele, *abstinence by mutual consent* (*amc*), was identified in a screen for defective pollen-tube reception (Boisson-Dernier et al., 2008). *amc* is a null allele of PEX13 and results in defective PTS1 import in pollen and eventual gametophytic lethality (Boisson-Dernier et al., 2008). In this chapter, I describe the characterization of physiological and molecular phenotypes of two new *pex13* alleles.

3.1 *pex13-1* is a weak peroxin mutant with reduced *PEX13* expression

To explore the genetic interactions of PEX13 (At3g07560) with mutants implicated in peroxisomal import and receptor recycling, we isolated *pex13-1*, a mutant from the Salk collection of sequence-indexed T-DNA lines (Alonso et al., 2003). I sequenced the *PEX13* coding sequence from *pex13-1* genomic DNA and found no mutations other than the T-DNA. The T-DNA is inserted in the *PEX13* 5′-UTR at position -46 relative to the initiator ATG (Figure 3.1). To test whether the insertion altered *PEX13* expression, I isolated RNA from wild type and *pex13-1* and performed

* Part of the work described in this chapter has been published (Ratzel et at., 2010).

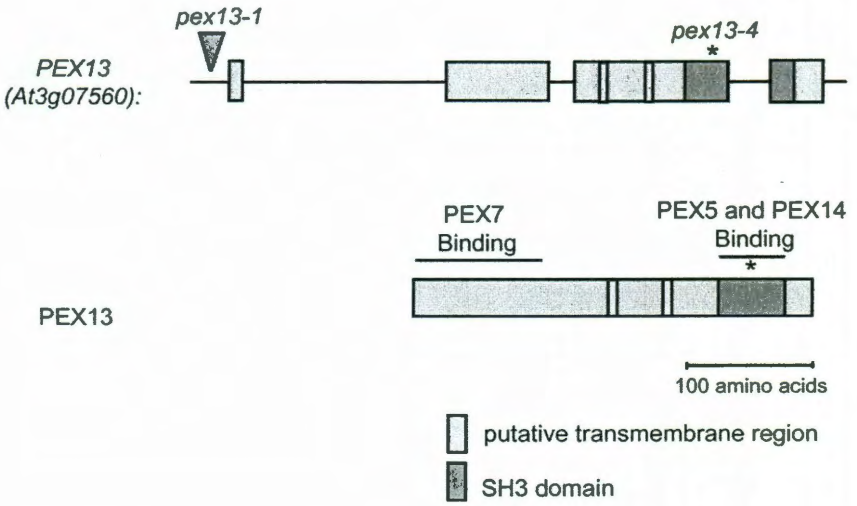

Figure 3.1 Schematic illustration showing *pex13* lesions and domains in PEX13. *PEX13* has four exons (blue boxes) separated by three introns (lines). The position of the *pex13-1* T-DNA insertion is indicated with a triangle. The position of the *pex13-4* lesion is marked with an asterisks. Putative transmembrane (yellow box) and SH3 (green box) domains are indicated. The binding sites of interacting proteins are indicated above the protein.

RNA gel blot analysis. I found that the *pex13-1* mutant had reduced *PEX13* mRNA levels compared to wild type (Figure 3.2C). Because the two previously characterized *pex13* mutants display phenotypes suggestive of peroxisome deficiency (Mano et al., 2006; Boisson-Dernier et al., 2008), I expected that this partial loss-of-function mutant also might be defective in peroxisomal function.

Sucrose dependence can be used to assay peroxisome function because oilseed plants, such as Arabidopsis, rely on acetyl-CoA derived from peroxisomal fatty-acid β-oxidation as a source of fixed carbon during post-germinative growth until photosynthesis is established (Baker et al., 2006). *pex* mutants often display growth defects on unsupplemented medium that can be at least partially restored by exogenous sucrose, including reduced hypocotyl elongation in the dark, like *pex5-1* (Zolman et al., 2000), and/or reduced root growth in the light, like *pex6-1* (Zolman and Bartel, 2004). I found that the *pex13-1* mutant was not sucrose dependent in the dark (Figure 3.2A), but appeared weakly sucrose dependent in the light (Figure 3.2B), suggesting that fatty acid β-oxidation was at most slightly impaired in this mutant.

IBA resistance is another measure of peroxisomal function. Genetic and biochemical evidence suggest that IBA is β-oxidized in peroxisomes to the active hormone IAA in a manner similar to fatty-acid β-oxidation (Zolman et al., 2000; Strader et al., 2010), and numerous mutants with peroxisomal defects, such as *pex5-1*, display IBA resistant primary root growth (Zolman et al., 2000). Similarly, peroxisomes are implicated in the β-oxidation of 2,4-DB to the auxinic compound 2,4-D (Hayashi et al., 1998). I found that the *pex13-1* mutant was not notably resistant to the inhibitory effects

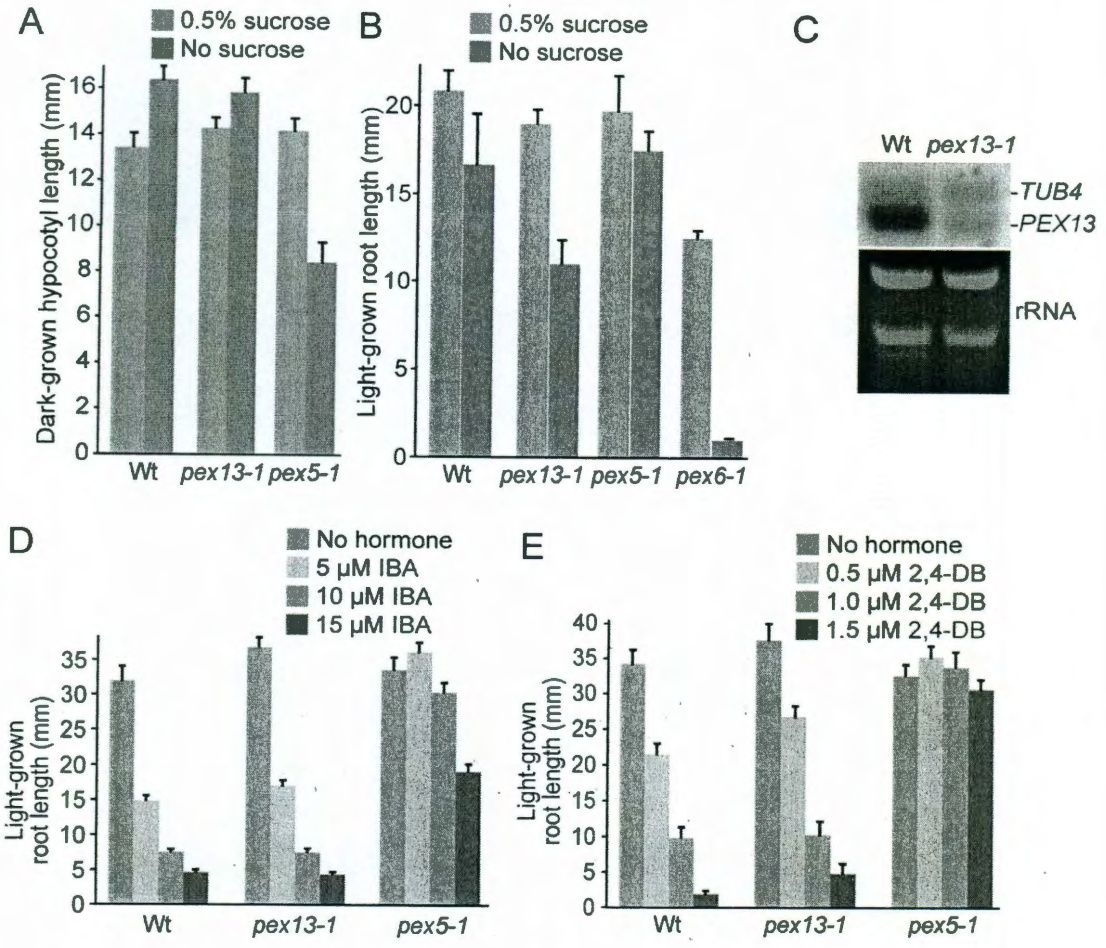

Figure 3.2 *pex13-1* is a T-DNA insertion allele that lacks typical *pex* phenotypes. (A, B) *pex13-1* is not sucrose dependent in the dark (A) but appears to be slightly sucrose dependent in the light (B). The dark assay shows mean hypocotyl lengths of 5-day-old Col-0 (Wt), *pex13-1*, and *pex5-1* seedlings grown on medium with and without 0.5% sucrose (A). The light assay shows mean root lengths of 8-day-old Col-0 (Wt), *pex13-1*, *pex5-1*, and *pex6-1* seedlings grown under yellow light on medium with and without 0.5% sucrose (B). (C) *pex13-1* has reduced accumulation of *PEX13* mRNA. RNA extracted from 10-day-old light-grown Col-0 (Wt) and *pex13-1* seedlings was subjected to RNA gel-blot analysis probed with *PEX13* and *TUB4* (top panel). The bottom panel shows the ethidium bromide-stained gel prior to transfer. (D, E) *pex13-1* responds similarly to wild type to IBA (D) and 2,4-DB (E). Mean root lengths of 8-day-old Col-0 (Wt), *pex13-1*, and *pex5-1* seedlings grown on medium supplemented with 0.5% sucrose and the indicated hormone under yellow-filtered light are shown. Error bars indicate standard errors of the means ($n \geq 12$).

of IBA or 2,4-DB on primary root elongation (Figure 3.2D, E), suggesting that IBA and 2,4-DB β-oxidation were not markedly impaired in this mutant.

To determine if *pex13-1* harbored any molecular phenotypes despite a lack of physiological phenotypes, I performed immunoblot analysis. Surprisingly, even with the reduced mRNA accumulation of *PEX13* in *pex13-1*, I did not detect reduced accumulation of PEX13 protein in seedling extracts (Figure 3.3). Furthermore, PEX5, PEX7, and PEX14 all accumulated at wild-type levels in *pex13-1* (Figure 3.3). To determine if *pex13-1* had a PTS2 processing defect, I probed with antibodies to the PTS2-containing proteins PMDH and thiolase. Like in wild type, both PMDH and thiolase were fully processed in *pex13-1* (Figure 3.3).

3.2 *pex13-4* is a strong peroxin mutant

To date, the *pex13* alleles that have been described are either weak alleles, such *pex13-1* (Ratzel et al., 2010) and *apm2* (Mano et al., 2006), which result in slightly defective peroxisomal function, or a null allele, *amc*, that is gametophytic lethal (Boisson-Dernier et al., 2008). Interestingly, none of these alleles result in mutants displaying intermediate phenotypes or exhibiting specific defects in protein partner binding. To dissect PEX13 interactions, I characterized a recently isolated *pex13* missense allele.

pex13-4 was identified in an EMS mutagenesis screen for sucrose dependent, IBA resistant seedlings by Marta Bjornson and Andrew Woodward. The mutation is a Glu to Lys mutation that lies in the region of the SH3 domain that in yeast PEX13 is required for PEX14 interaction (Pires et al., 2003). Because this mutation could render pex13-4

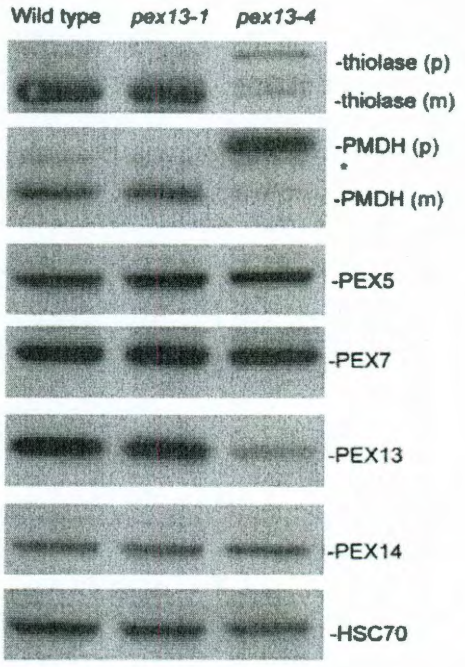

Figure 3.3 *pex13-4*, but not *pex13-1*, has decreased PEX13 accumulation and PTS2processing defects.

Protein extracts from eight 5-day-old seedlings grown in the light on medium with 0.5% sucrose were processed for sequential immunoblotting using the indicated antibodies. Precursor (p) and mature (m) proteins contain or lack, respectively, the N-terminal PTS2 peptide. α-HSC70 was used as a loading control. Positions of molecular mass markers are not indicated because the marker did not appear on the films. The asterisk indicates residual PEX7 immunoreactivity from a prior exposure of the filter.

incapable of forming a functional docking complex with PEX14, I compared the defects of this mutant to those of *pex13-1* and the previously described *pex5-1* (Zolman et al., 2000) and *pex6-1* (Zolman and Bartel, 2004) mutants.

Like many *pex* mutants, *pex13-4* was IBA resistant compared to wild type and *pex13-1* in primary root elongation (Figure 3.4A). *pex13-4* displayed greater IBA resistance than *pex5-1* or *pex6-1*, suggesting a stronger defect in peroxisomal function. Furthermore, *pex13-4* displayed dramatic sucrose dependence when grown in either the light or the dark (Figure 3.4B,C). Other sucrose dependent *pex* mutants, like *pex6-1*, germinate before arresting on medium lacking sucrose, but *pex13-4* appeared not to germinate, indicating a more severe defect (data not shown).

To examine the molecular phenotypes of the *pex13-4* mutant, I performed immunoblot analysis. Compared to wild-type and *pex13-1*, *pex13-4* had reduced accumulation of PEX13 (Figure 3.3) indicating that protein stability is disrupted by the mutation. Despite reduced PEX13 levels, PEX5, PEX7, and PEX14 accumulated to wild type levels in *pex13-4* (Figure 3.3).

Because Arabidopsis PTS2 proteins are processed via removal of the signal sequence to smaller, mature forms upon peroxisome entry by the protease DEG15, a PTS1 protein (Helm et al., 2007), immunoblotting can be used to indirectly monitor PTS1 and/or PTS2 matrix protein import defects. To determine if the reduced pex13-4 levels were accompanied by reduced peroxisomal import, I examined thiolase and PMDH processing. In wild-type and *pex13-1*, thiolase and PMDH were completely processed (Figure 3.3). In contrast, *pex13-4* seedlings displayed reduced PTS2 processing. The majority of thiolase and PMDH were unprocessed in *pex13-4* (Figure 3.3), indicating a

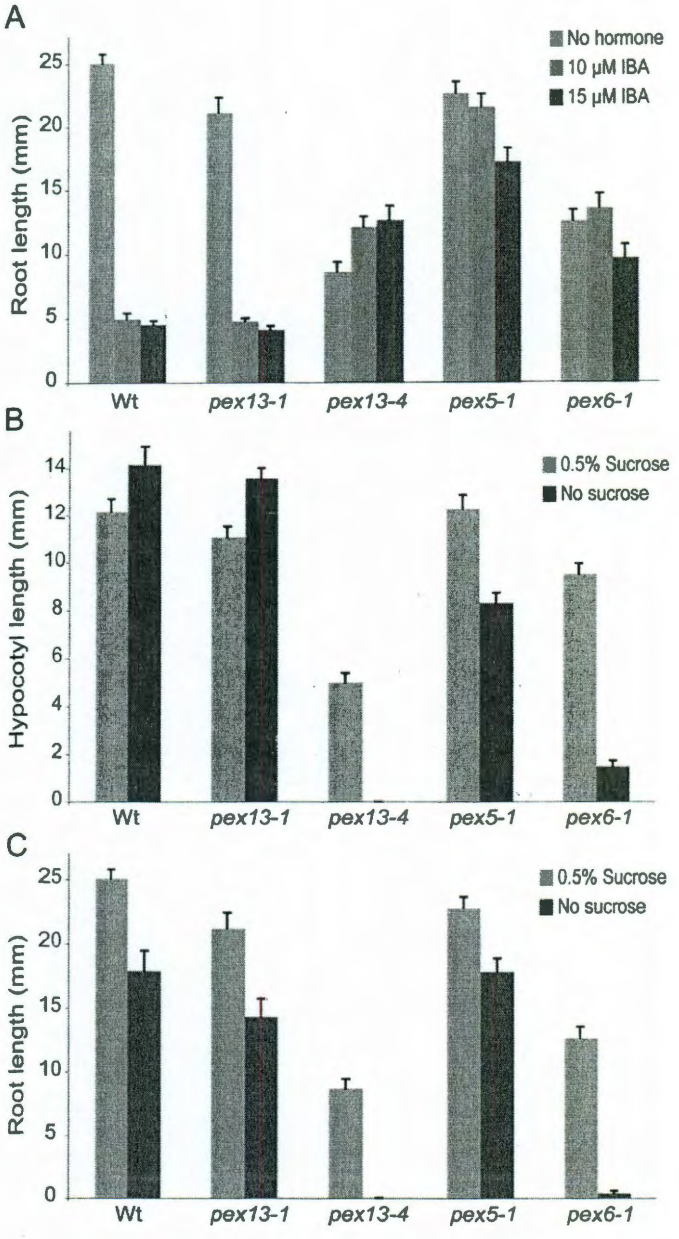

Figure 3.4 *pex13-4* is IBA resistant and sucrose dependent.
(A) *pex13-4* is IBA resistant. Mean root lengths of 8-day-old Col-0 (Wt), *pex13-1*, *pex13-4*, *pex5-1*, and *pex6-1* seedlings grown on medium supplemented with 0.5% sucrose and the indicated IBA concentration under yellow-filtered light are shown. pex13-4 is sucrose dependent in the dark (B) and in the light (C). The dark assay shows mean hypocotyl lengths of 5-day-old seedlings grown on medium with and without 0.5% sucrose (B). The light assay shows mean root lengths of 8-day-old seedlings grown under yellow light on medium with and without 0.5% sucrose (C). Error bars indicate standard errors of the means ($n \geq 12$) for panels (A) through (C).

strong defect in PTS1 and/or PTS2 import. In addition to reduced PTS2 processing, thiolase levels were generally reduced in *pex13-4* compared to wild type or *pex13-1* (Figure 3.3).

3.3 Conclusions

pex13-1 carries a T-DNA insertion in the 5′ UTR (Figure 3.1) and displays reduced accumulation of *PEX13* mRNA but without dramatic physiological phenotypes. Although the slight root elongation defect of light-grown *pex13-1* seedlings was rescued by sucrose, *pex13-1* did not display other physiological phenotypes typically associated with Arabidopsis *pex* mutants, such as sucrose-dependent growth in the dark, IBA resistance, or 2,4-DB resistance (Figure 3.2 and 3.4). Consistent with the lack of physiological phenotypes, *pex13-1* did not display detectable PTS2 processing defects (Figure 3.3).

In contrast, *pex13-4* is a missense allele displaying reduced accumulation of PEX13 (Figure 3.3), complete sucrose dependence in both the light and the dark (Figure 3.4B,C), strong IBA resistance (Figure 3.4A), and growth defects even when supplemented with sucrose (Figure 3.4). These physiological phenotypes are accompanied by nearly abolished PTS2 processing (Figure 3.3), suggesting reduced PTS2 and/or PTS1 import. Because the *pex13-4* mutation lies in the region of the SH3 domain required for PEX14 interaction in yeast (Pires et al., 2003), *pex13-4* phenotypes may reflect disrupted docking complex formation. Alternatively, the reduced PEX13 protein levels found in *pex13-4* may be insufficient to support efficient matrix protein

import. Examining interaction between PEX14 and pex13-4 versus PEX13 would aid in distinguishing between these possibilities (discussed in Chapter 8).

Chapter 4: *pex13-1* Enhances Early-Acting Peroxin Mutants*

Although the slight root elongation defect of light-grown *pex13-1* seedlings was rescued by sucrose, *pex13-1* did not display other physiological phenotypes typically associated with Arabidopsis *pex* mutants, such as sucrose-dependent growth in the dark, IBA resistance, or 2,4-DB resistance (Chapter 3). Consistent with the lack of physiological phenotypes, *pex13-1* did not display detectable PTS2 processing defects (Chapter 3). However, some weak *pex* mutants that lack striking phenotypes yield enhanced phenotypes when combined with other *pex* mutants. For example, although *pex22-1* and *pex7-1*, two weak *pex* alleles that also harbor T-DNA insertions upstream of the corresponding *PEX* gene coding sequences, lack severe phenotypes on their own; *pex22-1* dramatically enhances *pex4-1* defects and *pex7-1* dramatically enhances *pex5-1* defects (Woodward and Bartel, 2005b; Zolman et al., 2005). To determine whether the *pex13-1* lesion would enhance other *pex* mutants, we made a series of double mutants with *pex* alleles implicated in different aspects of peroxisomal import and recycling (Figure 1.1): receptor function (*pex5-1* and *pex5-10*), docking (*pex14-2*), ubiquitination of the receptor to initiate recycling (*pex4-1*), and receptor extraction from the peroxisome (*pex6-1*). In this chapter, I describe double mutants isolated with *pex13-1* and the early-acting mutants *pex5-1*, *pex5-10*, and *pex14-2*.

4.1 *pex13-1* enhances *pex5-1* sucrose dependence and IBA resistance

The *pex5-1* missense allele of the PEX5 (At5g56290) PTS1 receptor is moderately sucrose dependent in the dark but not notably sucrose dependent in the light,

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* The work described in this chapter has been published (Ratzel et al., 2010).

whereas the more severe *pex5-10* allele is completely sucrose dependent in both the light and the dark (Zolman et al., 2005; Ramón and Bartel, 2010). I found that the *pex5-1 pex13-1* double mutant was more sucrose dependent in both the light and the dark (Figure 4.1.A-C) and more IBA resistant in primary root growth (Figure 4.1.D, E) than either parent. Similarly, the *pex5-1 pex13-1* double mutant responded less than either parent to the stimulatory effect of IBA on lateral root production (Figure 4.1.F). I concluded from this enhancement that the reduced *PEX13* expression in the *pex13-1* allele (Figure 3.1C) further impaired peroxisomal function when peroxisome function was already compromised in the *pex5-1* single mutant. By contrast, I did not detect *pex5-10* enhancement by *pex13-1* in either sucrose dependence or IBA resistance (Figure 4.1A-F). Similarly, the weak *pex7-1* allele dramatically enhances *pex5-1* phenotypes (Woodward and Bartel, 2005b) but does not enhance *pex5-10* phenotypes (Ramón and Bartel, 2010), consistent with the possibility that *pex5-10* phenotypes are too severe to be enhanced with a slight reduction in a second early-acting peroxin.

In Arabidopsis, PTS2 processing can serve as an indirect measure of PTS2 and/or PTS1 import. I examined PTS2 processing using antibodies raised against a thiolase isoform (PED1, Lingard et al., 2009) and a peroxisomal malate dehydrogenase isoform (PMDH2, Pracharoenwattana et al., 2007). As previously described (Woodward and Bartel, 2005b; Zolman et al., 2005; Ramón and Bartel, 2010), I found moderate and severe PTS2 processing defects in *pex5-1* and *pex5-10*, respectively (Figure 4.2). Consistent with the trend in physiological phenotypes, *pex13-1* processed PTS2 proteins normally and slightly enhanced *pex5-1*, but not *pex5-10*, processing defects in the light and in the dark (Figure 4.2).

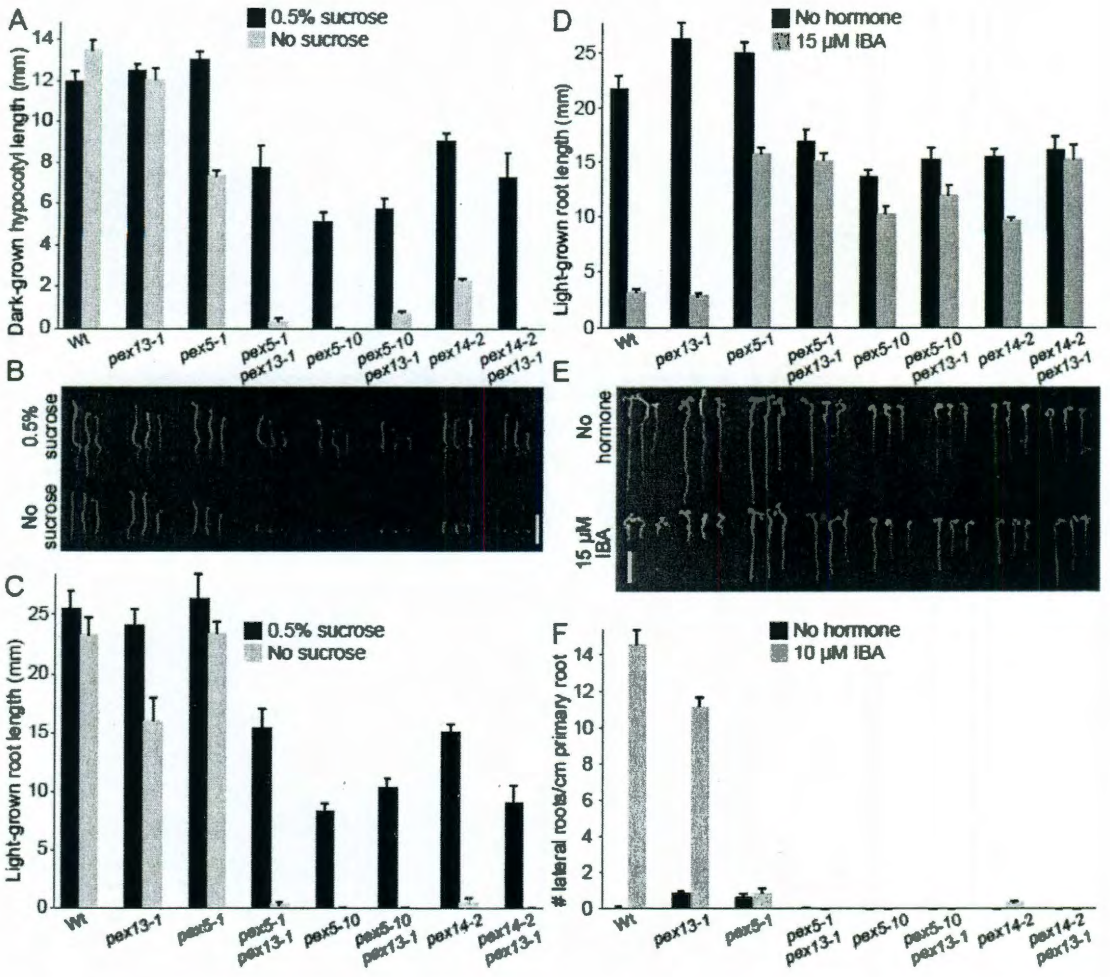

Figure 4.1 *pex13-1* enhances some physiological phenotypes of early-acting *pex* mutants.

(A-C) *pex13-1* enhances the sucrose dependence of early-acting *pex* mutants. Mean hypocotyl lengths of 5-day-old seedlings grown in the dark on medium with and without 0.5% sucrose are shown (A). Three seedlings representing the range of phenotypes for each line were photographed (B). Mean root lengths of 8-day-old seedlings grown in yellow light on medium with and without 0.5% sucrose are shown (C). (D-E) *pex13-1* enhances IBA resistance of early-acting *pex* mutants. Mean root lengths of 8-day-old seedlings grown on 0.5% sucrose-supplemented medium with and without 15 µM IBA under yellow light are shown (D). Three representative seedlings from each line were photographed (E). (F) *pex13-1* does not impact the IBA-resistance of early-acting pex mutants in a lateral root formation assay. Four-day-old seedlings were transferred to medium with or without 10 µM IBA for an additional 4 days under yellow light, after which root lengths were measured and primary roots were counted. Error bars in panels A, C, D, and F represent standard errors of the means ($n \geq 12$). Scale bars in B and E = 10 mm.

4.2 *pex13-1* enhances *pex14-2* peroxisomal defects

PEX13 functions in a docking complex with a second membrane peroxin, PEX14 (At5g62810). *pex14-2*, which is disrupted by a T-DNA insertion in the first exon (Monroe-Augustus et al., 2010), is IBA resistant and sucrose dependent (Figure 4.1). To examine genetic interactions between *PEX13* and *PEX14*, we isolated the *pex14-2 pex13-1* double mutant. *pex13-1* enhanced *pex14-2* sucrose dependence and IBA resistance: the double mutant was completely dependent on sucrose for seedling growth in both the light and the dark (Figure 4.1A-C) and was completely resistant to the inhibitory effects of 15 μM IBA on root elongation (Figure 4.1D, E).

PTS2 processing in *pex14-2* seedlings was defective. Light- and dark-grown *pex14-2* seedlings displayed a mild thiolase-processing defect, and approximately half of the PMDH was unprocessed in light-grown seedlings (Figure 4.2). Similar to the enhancement of physiological defects (Figure 4.1), *pex13-1* clearly enhanced the PTS2 processing defects of *pex14-2* seedlings in both light- and dark-grown seedlings (Figure 4.2).

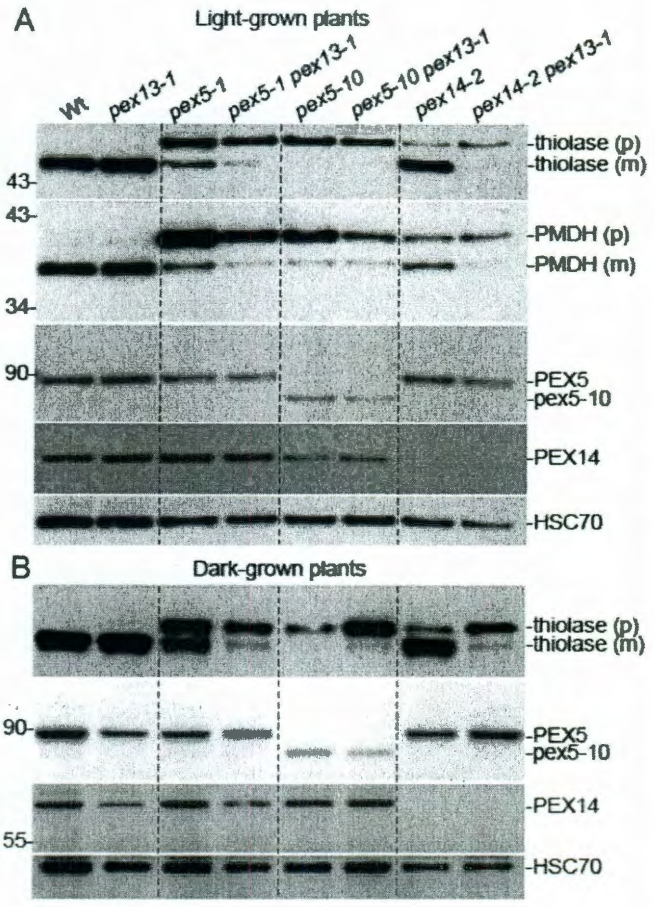

Figure 4.2 *pex13-1* does not exhibit PTS2 processing defects but enhances the PTS2 processing defects of *pex14-2*.
Protein extracts from eight 5-day-old seedlings grown in the light (A) or the dark (B) on medium with 0.5% sucrose were processed for immunoblotting and probed sequentially with the indicated antibodies. Precursor (p) and mature (m) proteins contain or lack, respectively, the N-terminal PTS2 peptide. α-HSC70 was used as a loading control, and the positions of molecular mass markers (in kDa) are indicated on the left.

4.3 Conclusions

The sucrose dependence and IBA resistance of *pex* mutants that are compromised early in matrix protein import (*pex5-1* and *pex14-2*) were enhanced by *pex13-1* (Figure 4.1). In addition, growth of *pex5-1 pex13-1* and *pex14-2 pex13-1* was more impaired than the parental mutants, suggesting that reduced *PEX13* expression was sufficient to reduce peroxisome function in these backgrounds. The clear enhancement of *pex5-1* and *pex14-2* defects by *pex13-1* indicate that PEX13 function is reduced in *pex13-1* even though whole seedling extracts did not reveal a dramatic reduction in PEX13 protein accumulation (Figure 3.2). These results support the intuitive hypothesis that combining two *pex* mutations magnifies the loss of peroxisomal function.

Interestingly, the early-acting double mutants had differential PTS2 processing defects in combination with *pex13-1*. *pex13-1* clearly magnified *pex14-2* PTS2 processing defects (Figure 4.2), suggesting that multiple lesions in the docking complex yielded synergistic import impediments. In contrast, PTS2 processing defects in the *pex5* mutants, which were already quite severe in the single mutants, were either not enhanced (*pex5-10*) or only slightly enhanced (*pex5-1*) by *pex13-1* (Figure 4.2). However, the clear enhancement of the *pex5-1* physiological defects by *pex13-1* (Figure 4.1), imply that reducing *PEX13* function had synergistic deleterious effects on *pex5-1* peroxisomes.

Chapter 5: *pex13-1* Suppresses Late-Acting Peroxin Mutants*

I identified and characterized *pex13-1*, a T-DNA insertion mutant in *PEX13* 5′ UTR, that lacks notable physiological or molecular defects (Chapter 3) and enhances *pex14-2* defects (Chapter 4). To determine whether the *pex13-1* lesion would enhance late-acting *pex* mutants, I made double mutants with *pex* alleles implicated in different aspects of peroxisomal receptor recycling (Figure 1.1): ubiquitination of the receptor to initiate recycling (*pex4-1*), and receptor extraction from the peroxisome (*pex6-1*). In contrast to the enhancement of early-acting *pex* mutants, I found that *pex13-1* suppressed several peroxisome-defective phenotypes of two mutants defective in proteins that act in receptor recycling after cargo translocation (late-acting *pex* mutants).

5.1 *pex13-1* suppresses *pex4-1* and *pex6-1* sucrose dependence and IBA resistance

I examined the impact of *pex13-1* on late-acting *pex* mutants by assaying sucrose dependence and IBA resistance of *pex4-1 pex13-1* and *pex6-1 pex13-1* double mutants. Arabidopsis PEX4 (At5g25760) is orthologous to the yeast ubiquitin-conjugating enzyme that is necessary for mono-ubiquitination and recycling of PEX5 (Platta et al., 2004). PEX6 (At1g03000) is orthologous to an AAA ATPase that is required for recognition and removal of the mono-ubiquitin-tagged PEX5 from the peroxisome (Platta et al., 2005). The Arabidopsis *pex4-1* and *pex6-1* missense alleles are both sucrose dependent and IBA resistant (Zolman and Bartel, 2004; Zolman et al., 2005).

I was surprised to find that *pex13-1* restored the hypocotyl and root elongation defects of *pex4-1* and *pex6-1* seedlings grown on sucrose-containing medium (Figure

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* The work described in this chapter has been published (Ratzel et al., 2010).

5.1A-C). Moreover, *pex13-1* completely rescued the hypocotyl elongation defects displayed by *pex4-1* and *pex6-1* seedlings grown in the dark in the absence of sucrose (Figure 5.1A, B). Both double mutants were less sucrose dependent in the light than either *pex4-1* or *pex6-1* single mutants, although the suppression was incomplete in light-grown seedlings (Figure 5.1C). Similarly, I found that *pex13-1* partially restored IBA responsiveness to *pex4-1* in both primary root growth inhibition and lateral root promotion (Figure 5.1D-F). In contrast, the *pex6-1 pex13-1* double mutant remained highly resistant to the inhibitory effects of IBA on root elongation and to the stimulatory effects of IBA on lateral root formation (Figure 5.1D-F).

To characterize the sensitivity of *pex6-1* to *PEX13* levels, I tested the dominance of the *pex13-1* allele in the *pex6-1* background. I assayed a *pex6-1* line segregating for the *pex13-1* T-DNA insertion for growth on medium lacking sucrose and then assessed seedling genotypes to determine how many *pex13-1* copies were required to confer sucrose independence. *pex6-1* with two copies of wild-type *PEX13* (+/+) displayed short hypocotyls on medium lacking sucrose, similar to the *pex6-1* line. Homozygous *pex13-1* (-/-) conferred sucrose independence to *pex6-1* (Figure 5.2), as expected from the sucrose independence of the homozygous *pex6-1 pex13-1* mutant (Figure 5.1A). *pex6-1* seedlings with only one copy of the *pex13-1* insertion (+/-) had hypocotyl lengths intermediate between *pex6-1* and *pex6-1 pex13-1* seedlings (Figure 5.2). Because *pex13-1* seedlings fail to accumulate wild-type levels of *PEX13* mRNA (Figure 1.1C), this semidominance likely reflects haploinsufficiency and indicates that peroxisome function is sensitive to *PEX13* levels in the *pex6-1* mutant in a dosage-dependent manner.

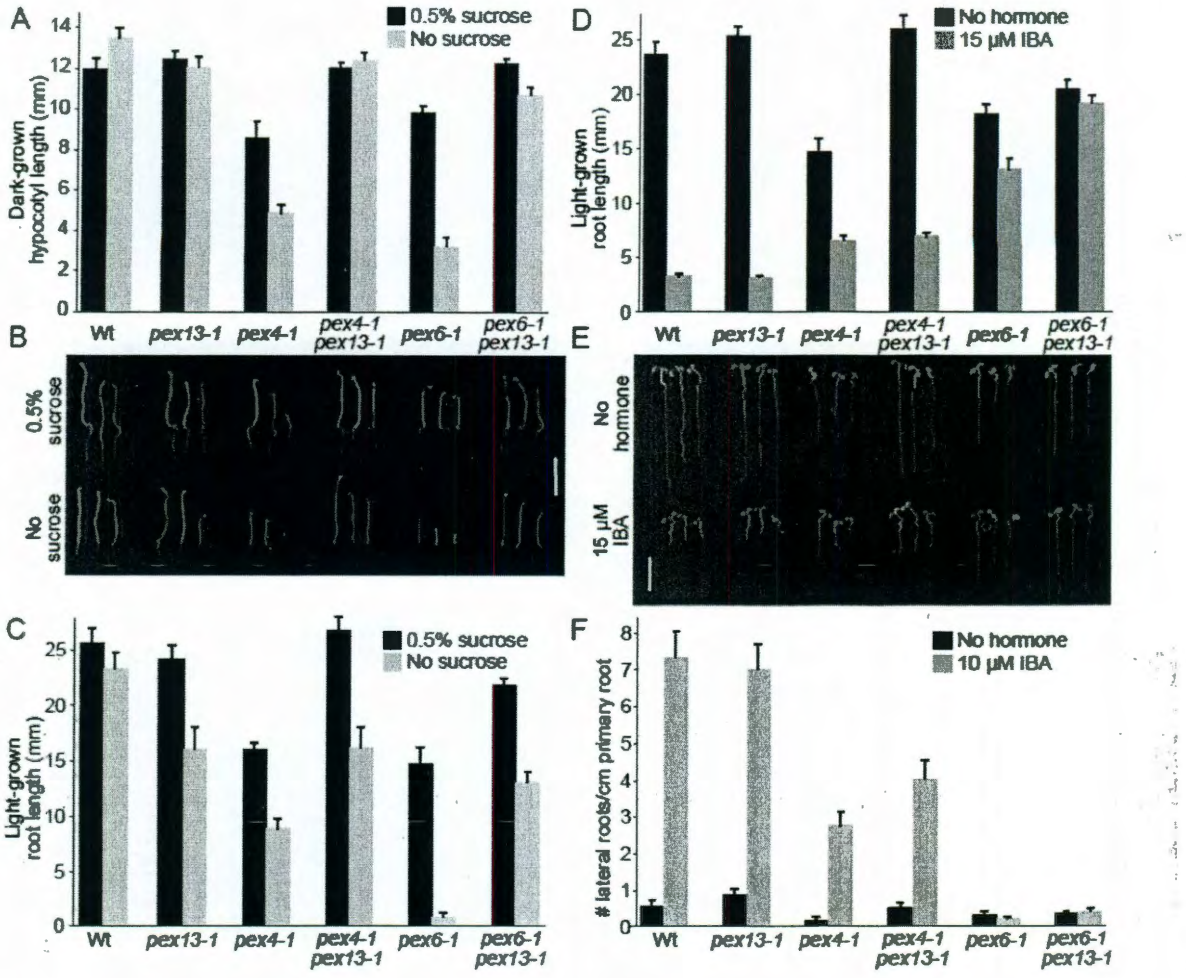

Figure 5.1 *pex13-1* suppresses some physiological phenotypes of late-acting *pex* mutants.

(A-C) *pex13-1* suppresses the sucrose dependence of late-acting *pex* mutants in the dark (A, B) and the light (C). (D-E) *pex13-1* partially suppresses the IBA resistance of *pex4-1*, but not *pex6-1*. Seedlings were assayed as in the legend to Figure 4.1. Error bars in panels A, C, D, and F indicate standard errors of the means ($n \geq 12$). Scale bars in B and E = 10 mm.

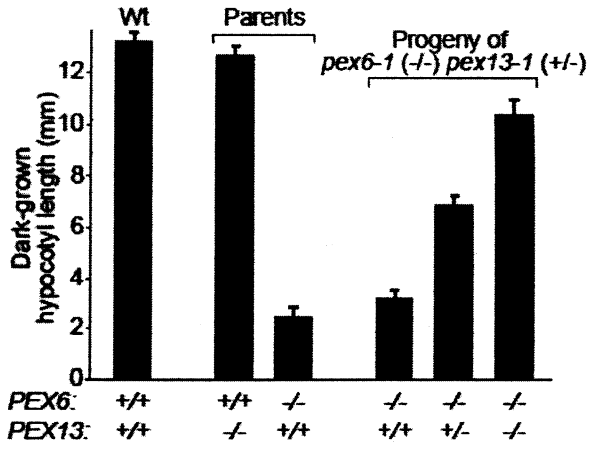

Figure 5.2 Dosage-dependent suppression of *pex6-1* sucrose requirement by *pex13-1*.

Progeny from a parental line homozygous for *pex6-1* and heterozygous for *pex13-1* were grown for 1 day in the light followed by 4 days in the dark on medium lacking sucrose and hypocotyl lengths were measured. Seedling genotypes were determined using PCR to determine the allelic state at *pex13-1*. Average length for *PEX13/PEX13* (+/+; $n = 4$), *PEX13/pex13-1* (+/-; $n = 7$), and *pex13-1/pex13-1* (-/-; $n = 10$) seedlings were determined. Error bars indicate standard errors of the means ($n \geq 12$ for controls).

5.2 *pex13-1* does not alter levels of other peroxins

To examine the possibility that reduced *PEX13* levels might impact levels of other peroxins in the double mutants, I conducted immunoblotting experiments with extracts from light- and dark-grown seedlings. *pex13-1* did not alter PEX5, PEX6, PEX7, or PEX14 protein levels in the *pex4*, *pex5*, *pex6*, or *pex14* mutants (Figure 4.2 and 5.3). The two late-acting *pex* mutants, *pex4* and *pex6*, can be distinguished by their PEX5 levels; PEX5 levels are normal in *pex4-1* (Zolman et al., 2005), but are reduced in *pex6-1* (Zolman and Bartel, 2004). My observation that the *pex6-1* defect in PEX5 accumulation was maintained in light- and dark-grown *pex6-1 pex13-1* seedlings (Figure 5.3A, C) indicates that *pex13-1* did not restore *pex6-1* physiological defects (Figure 5.1A-C) by restoring PEX5 to wild-type levels.

5.3 *pex13-1* partially rescues certain *pex4-1* and *pex6-1* PTS2 processing defects

Because *pex13-1* restored *pex4-1* sucrose independence and IBA responses, I used immunoblotting to examine PTS2 processing to determine whether physiological rescue was accompanied by restored matrix protein import. *pex4-1* seedlings display mild PTS2 processing defects (Zolman et al., 2005). Unlike *pex14-2 pex13-1* (Figure 4.2), I found that the *pex4-1* and *pex6-1* PTS2 processing defects were not enhanced by *pex13-1*. In fact, the slight thiolase defects of *pex4-1* and *pex6-1* appeared to be rescued by *pex13-1*, whereas the *pex4-1* and *pex6-1* PMDH processing defects were not convincingly altered by *pex13-1* (Figure 5.3A). The difference in PTS2 processing restoration of thiolase and PMDH suggests either that some PTS2 proteins are preferentially imported or perhaps that unprocessed PTS2 proteins have different stabilities in different mutant backgrounds.

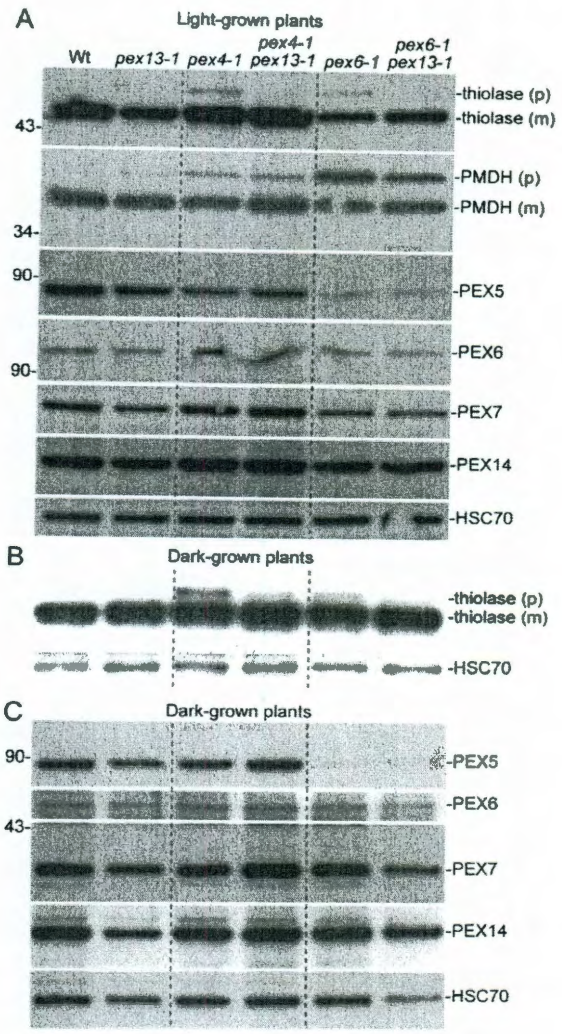

Figure 5.3 *pex13-1* does not dramatically alter PTS2 processing defects or peroxin levels in *pex4-1* or *pex6-1*.
Protein extracts from eight 5-day-old seedlings grown in the light (A) or the dark (B, C) on medium with 0.5% sucrose were processed for immunoblotting using the indicated antibodies. Precursor (p) and mature (m) proteins contain or lack, respectively, the N-terminal PTS2 peptide. α-HSC70 was used as a loading control, and the positions of molecular mass markers (in kDa) are indicated on the left.

5.4 The distribution of the PEX5 and PEX7 receptors is similar in *pex13-1* and wild type

PTS2 processing is an indirect measure of peroxisomal import that can result from reduced import of the PTS2 protein, reduced import of DEG15, the PTS1-tagged PTS2 processing protease, or both. To directly assess PTS1 and PTS2 import in the *pex13-1* mutant, I fractionated seedling extracts into cytosolic and organellar fractions and monitored peroxins and matrix proteins in the resultant fractions. In both wild type and *pex13-1*, the membrane peroxins PEX14 and PEX13 and the mitochondrial ATPase are clearly localized to the organelle fraction (P, Figure 5.4) demonstrating the effectiveness of the isolation. In both wild type and *pex13-1*, the ratio of protein found in the organelle (P) versus the cytosolic (S) fraction was higher for PEX5 than for PEX7, and both receptors were found in both fractions, consistent with the idea that these receptors cycle in and out of the peroxisome. I also examined the distribution of peroxisomal matrix proteins in these fractions, including the PTS1 protein malate synthase (MLS), the PTS2 protein PMDH, and catalse, which lacks a typical PTS (Oshima et al., 2008). Although the ratio of peroxisomal to cytosolic catalase appeared to be slightly reduced *pex13-1* compared to wild type, the ratio of peroxisomal to cytosolic MLS and PMDH did not appear to be altered, suggesting that the *pex13-1* mutation had slight, if any deleterious effects on PTS1 or PTS2 protein import in an otherwise wild-type background.

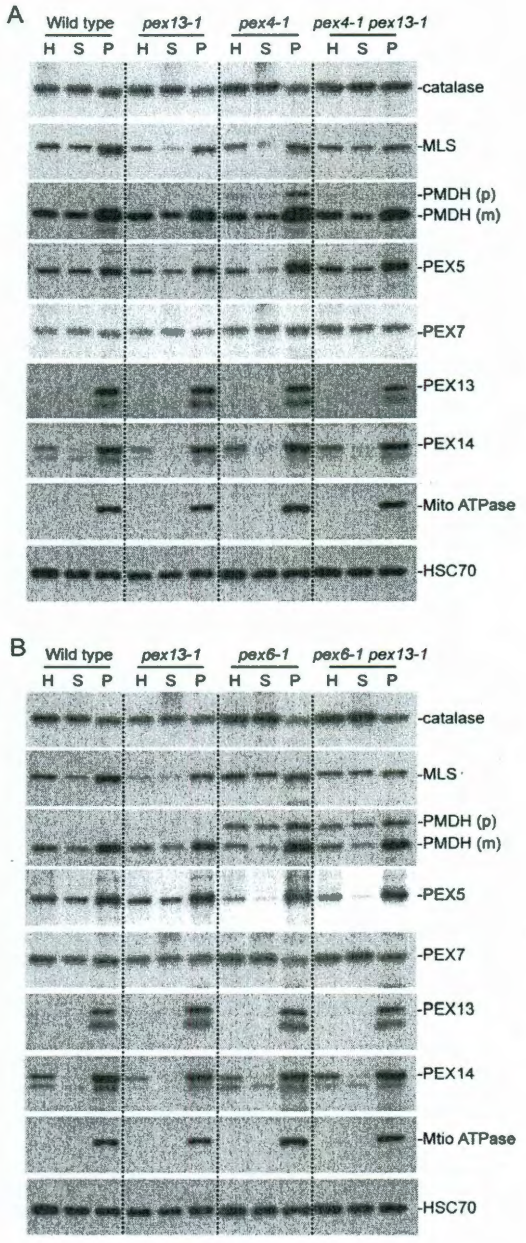

Figure 5.4 *pex13-1* alters PEX5 localization in *pex4-1* and does not rescue *pex6-1* matrix protein import defects.

(A, B) Whole seedling homogenates from 5-day-old light-grown seedlings of the indicated genotypes were separated by centrifugation into soluble and organellar pellet fractions. For each sample, 1% of the total homogenate (H), 1% of the soluble fraction (S) and 25% of the pellet fraction (P) were separated using SDS-PAGE and processed for sequential immunoblotting using the indicated antibodies. The mitochondrial membrane complex V subunit α (mito ATPase) and cytosolic HSC70 were used as organellar and cytosolic controls, respectively. Precursor (p) and mature (m) proteins contain or lack, respectively, the N-terminal PTS2 peptide. Positions of molecular mass markers (in kDa) are indicated on the left.

5.5 *pex4-1* over-accumulates PEX5 in the peroxisome and *pex13-1* alleviates this accumulation

Although total PEX5 levels are normal in *pex4-1* (Zolman et al., 2005), I found that the relative PEX5 distribution was shifted from the cytosolic fraction to the peroxisomal fraction in the *pex4-1* mutant (Figure 5.4A). This altered distribution supports the hypothesis that Arabidopsis PEX4, like PEX4 in other organisms (Platta et al., 2007; Grou et al., 2008), provides the ubiquitin that is needed to retrotranslocate PEX5 from the peroxisome. Interestingly, *pex13-1* appeared to restore the cytosolic/peroxisomal ratio of PEX5 in the *pex4-1 pex13-1* double mutant (Figure 5.4A).

In spite of the reduced level of cytosolic PEX5 in *pex4-1*, fractionation did not reveal dramatic defects in MLS or PMDH import in *pex4-1* (Figure 5.4A). This result is consistent with previous data showing that *pex4-1* lacks notable defects in GFP-PTS1 or PTS2-GFP localization (Zolman et al., 2005). However, the small fraction of PMDH that was unprocessed was largely organellar in *pex4-1* (Figure 5.4A), suggesting that this *pex4-1* PTS2 processing defect resulted from insufficient DEG15 import or activity rather than reduced PTS2 import.

5.6 *pex6-1* and *pex6-1 pex13-1* have reduced PTS1 and PTS2 import and reduced PEX7 accumulation in the peroxisome

Total PEX5 levels are reduced in *pex6-1* (Zolman and Bartel, 2004). I found that the remaining PEX5 in *pex6-1* was disproportionately present in peroxisomal fraction (Figure 5.4B). The *pex13-1* lesion did not appear to alter the aberrant receptor localization in the *pex6-1* mutant (Figure 5.4B). *pex6-1* receptor localization defects

were accompanied by cytosolic accumulation of the three monitored matrix proteins, catalase, MLS, and PMDH (Figure 5.4B).

Unprocessed PMDH was found in both the cytosolic and the peroxisomal fraction in *pex6-1* (Figure 5.4B), indicating that the *pex6-1* PTS2 processing defects reflect both PTS2 and PTS1 import defects. The *pex6-1* matrix protein import defects were not restored in the *pex6-1 pex13-1* double mutant.

5.7 *pex4-1 pex6-1* displays enhanced peroxisomal defects that are partially suppressed by *pex13-1*

My results demonstrate that the physiological defects of the *pex4-1* and *pex6-1* mutants, which are disrupted in late-acting peroxin genes, were partially suppressed by the reduced *PEX13* levels in the *pex13-1* mutant (Figure 5.1). To assess whether *pex13-1* could suppress a more severe block in late-acting peroxin function, we generated the *pex4-1 pex6-1* double mutant and the *pex4-1 pex6-1 pex13-1* triple mutant.

As expected, the *pex4-1 pex6-1* double mutant displayed increased sucrose dependence compared to either parent when grown in the dark (Figure 5.5A) or in the light (data not shown). Moreover, even when grown on sucrose-containing medium, the *pex4-1 pex6-1* double mutant had reduced hypocotyl length compared to either single mutant (Figure 5.5). These physiological defects were accompanied by an enhanced impairment in PTS2 processing suggestive of a more severe matrix protein import block; the *pex4-1 pex6-1* double mutant displayed a lower ratio of processed to unprocessed thiolase and PMDH than either parent (Figure 5.6).

Adding the *pex13-1* lesion partially suppressed the sucrose dependence of dark-grown *pex4-1 pex6-1* seedlings (Figure 5.5A). In addition, the hypocotyl growth defect

exhibited by the *pex4-1 pex6-1* double mutant even when sucrose supplemented was slightly ameliorated by *pex13-1* (Figure 5.5). This physiological suppression was not accompanied, however, by restored IBA sensitivity (Figure 5.5B) or PTS2 processing (Figure 5.6) in the *pex4-1 pex6-1 pex13-1* triple mutant, implying that the partial restoration of sucrose independence did not result from improved matrix protein import.

5.8 *pex4-1* restores PEX5 accumulation in *pex6-1*

One explanation for the *pex6-1* defects is that PEX5 is degraded rather than recycled when PEX6 cannot assist in removing PEX5 from the peroxisomal membrane (Zolman and Bartel, 2004), as suggested for yeast *pex6* mutants (Kiel et al., 2005a). PEX5 degradation in *pex6-1* may require a PEX4-dependent ubiquitination step, consistent with the observation that PEX5 levels are not appreciably reduced in *pex4-1* (Zolman et al., 2005). To determine whether PEX4 is required to reduce PEX5 levels in the *pex6-1* background, I performed immunoblot analysis in the *pex4-1 pex6-1* double mutant and found that *pex4-1* restored PEX5 levels in *pex6-1* (Figure 5.6). This epistatic relationship is consistent with PEX5 recycling models in which PEX4 acts prior to PEX6 (reviewed in Erdmann and Schliebs, 2005). Notably, restoration of PEX5 levels was accompanied by enhancement rather than restoration of the peroxisome deficient phenotypes in the *pex4-1 pex6-1* double mutant (Figure 5.5).

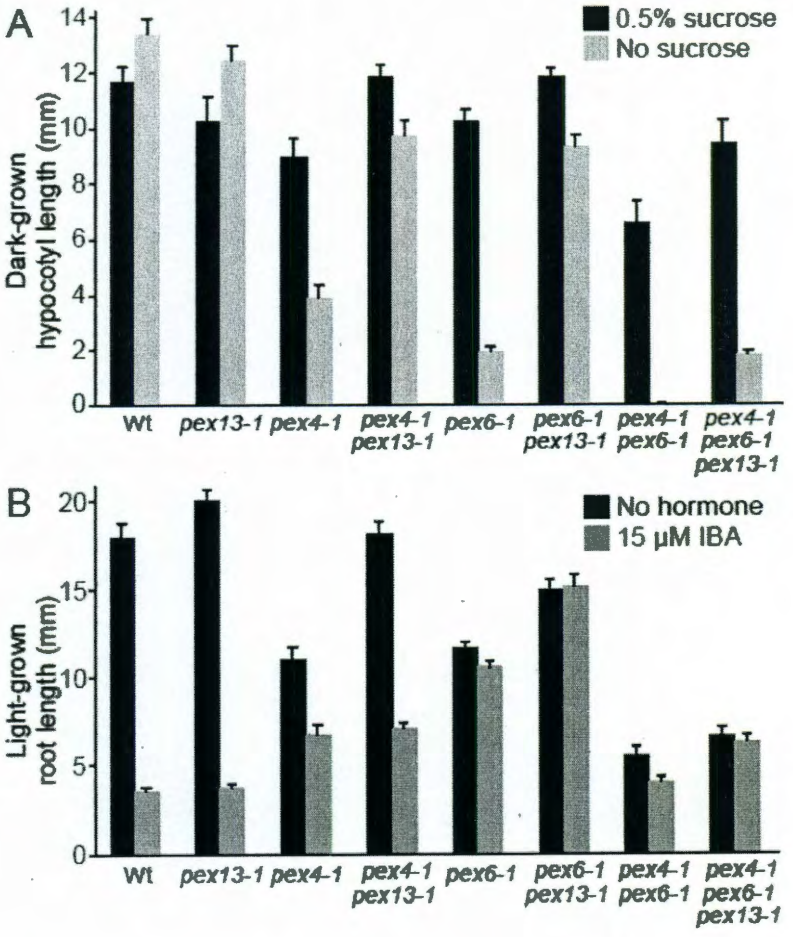

Figure 5.5 The *pex4-1 pex6-1* double mutant has enhanced sucrose dependence and IBA resistance and is partially suppressed by *pex13-1*.
(A, B) Seedlings were assays as in the legend to Figure 5.1 for sucrose dependence in the dark (A) or IBA resistance in the light (B). Error bars indicate standard errors of the means ($n \geq 12$).

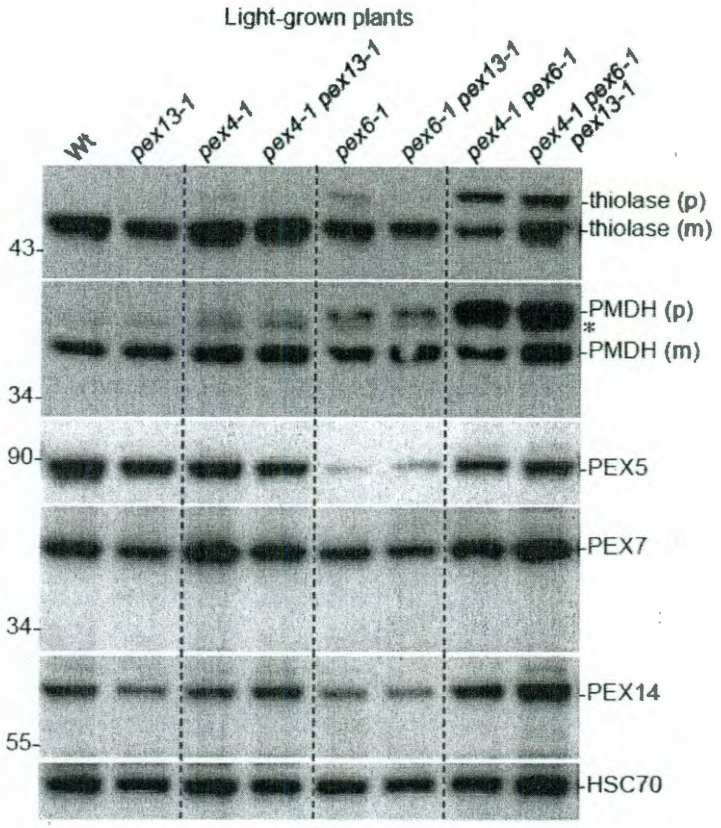

Figure 5.6 *pex4-1* restores PEX5 levels in *pex6-1*, and *pex13-1* does not suppress the enhanced PTS2 processing defect in the *pex4-1 pex6-1* double mutant.
Protein extracts from eight 5-day-old seedlings grown in the light on medium with 0.5% sucrose were processed for sequential immunoblotting using the indicated antibodies. Precursor (p) and mature (m) proteins contain or lack, respectively, the N-terminal PTS2 peptide. An asterisk indicates residual PEX7 signal from a previous hybridization. α-HSC70 was used as a loading control, and the positions of molecular mass markers (in kDa) are indicated on the left.

5.9 Conclusions

In contrast to the *pex13-1* enhancement of early-acting *pex* mutants (Chapter 4), we were surprised to find that *pex13-1* fully restored sucrose independence to the late-acting *pex4-1* and *pex6-1* mutants (Figure 5.1), and restored partial sucrose independence to the severe *pex4-1 pex6-1* double mutant (Figure 5.5). Moreover, the growth defects displayed by *pex4-1, pex6-1*, and *pex4-1 pex6-1* even when supplied with sucrose were partially suppressed by the *pex13-1* lesion. However, this physiological restoration was accompanied by only minimal if any suppression of PTS2 processing defects in the double mutants (Figure 5.3, 5.4) and the mild *pex6-1* PTS1 and PTS2 import defects were not noticeably suppressed by *pex13-1* (Figure 5.4), suggesting that the physiological suppression does not simply reflect restored matrix protein import in these mutants.

How do reduced *PEX13* levels suppress the physiological defects of *pex4-1*, *pex6-1*, and *pex4-1 pex6-1* mutants? It seems unlikely that when enough import is blocked by additive *pex* mutations, peroxisomal enzymes build up to sufficiently high concentrations in the cytosol so that they partially perform their functions even though mislocalized, because *pex13-1* does not enhance the PTS2 processing defects or matrix protein import defects of the *pex4-1* or *pex6-1* mutants (Figure 5.3). Moreover, the possibility of mislocalized but metabolically functional matrix enzymes is difficult to reconcile with the phenotypes of the *pex5-1 pex13-1*, *pex14-2 pex13-1* (Chapter 4), and *pex4-1 pex6-1* mutants described here or previously characterized double *pex* mutants, including *pex4-1 pex22-1* (Zolman et al., 2005) and *pex5-1 pex7-1* (Woodward and Bartel, 2005b), which show enhanced physiological defects accompanied by enhanced PTS2 processing defects.

We speculate that reducing *PEX13* levels might suppress *pex4-1* and *pex6-1* mutant phenotypes by restoring the balance between import and export of the peroxisomal receptor PEX5, and/or the balance between import and degradation of certain peroxisomal proteins (Figure 5.7). This hypothesis is consistent with the possibility that the physiological defects of *pex4-1* and *pex6-1* result primarily from defects in receptor recycling or peroxisomal matrix protein degradation rather than from defects in matrix protein import. Indeed, the barely detectable *pex4-1* matrix protein import defects do not seem commensurate with the severe sucrose dependence and IBA resistance displayed by the *pex4-1* mutant (Zolman et al., 2005). *pex4-1 pex22-1* and *pex6-1* mutants have defects in degrading certain matrix proteins (Lingard et al., 2009), suggesting that matrix proteins might exit the peroxisome for cytosolic degradation using some of the same machinery used by PEX5 to exit the peroxisome. Perhaps *pex13-1* reduces detrimental effects of undegraded matrix proteins by slowing matrix protein import. Moreover, *pex6-1* has reduced PEX5 levels (Zolman and Bartel, 2004). *PEX6* mutations also decrease PEX5 levels in the yeast *Pichia pastoris* (Collins et al., 2000) and mammals (Dodt and Gould, 1996). This conservation of a quality control mechanism by which PEX5 is degraded when recycling efficiency is reduced (Kiel et al., 2005a; Kiel et al., 2005b; Platta et al., 2007) is consistent with the possibility that PEX5 accumulation in the peroxisomal membrane has detrimental effects on peroxisome physiology. Perhaps *pex13-1* reduces the detrimental effects caused by PEX5 buildup in the membrane by decreasing the efficiency of PEX5 targeting to the membrane. In support of this possibility, we found restored cytosolic PEX5 in the *pex4-1 pex13-1* mutant (Figure 5.4A).

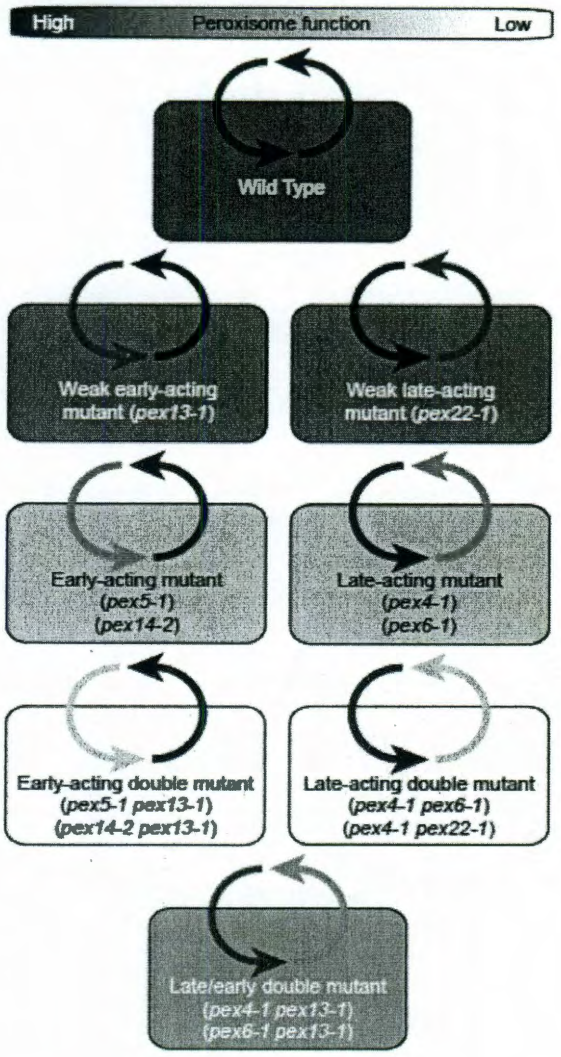

Figure 5.7 Balance model of peroxisomal import and export in *pex* mutants. Peroxisomal import and export occurs in a cyclical process in wild type (Figure 1.1). Early-acting *pex* mutants, such as *pex5-1* and *pex14-2*, are defective in matrix protein import. *pex13-1* has no detectable import defect but enhances other early-acting *pex* mutants, dramatically impairing peroxisome function in the *pex5-1 pex13-1* and *pex14-2 pex13-1* double mutants. Late-acting mutants have defects in retrotranslocating the PEX5 receptor and stabilize certain peroxisomal enzymes, perhaps because of similar export defects. *pex22-1* lacks detectable physiological defects but enhances the *pex4-1* late-acting *pex* mutant. Similarly, the late-acting *pex4-1 pex6-1* double mutant has enhanced peroxisomal defects. Unlike either early-acting or late-acting double mutants, combining a late-acting mutant with *pex13-1* partially restores peroxisome function, suggesting that slightly reducing import efficiency can partially ameliorate the detrimental effects of decreased peroxisomal export

Chapter 6: *pex13-1* has Contrasting Effects on Two *pex7* Alleles

In Chapters 4 and 5, I described a series of tests that can distinguish between early- and late-acting *pex* mutants. PEX7 is the PTS2 receptor (Woodward and Bartel, 2005b) and little is known about the interactions required for recycling of PEX7. Because the role of PEX7 in receptor recycling is incompletely understood, I isolated and characterized *pex13-1* double mutants with two *pex7* alleles. *pex7-1* is a T-DNA insertion in the 5′ UTR that results in reduced PEX7 accumulation (Woodward and Bartel, 2005b), and *pex7-2* is a missense allele that results in both PEX7 and PEX5 instability (Ramón and Bartel, 2010). In contrast to the clear division between early- and late-acting mutant phenotypes, *pex7 pex13-1* phenotypes vary depending on the *pex7* allele examined. I found that *pex7-2 pex13-1* displayed enhanced peroxisome-defective phenotypes and *pex7-1 pex13-1* displayed reduced peroxisome-defective phenotypes.

6.1 *pex13-1* enhances sucrose dependence and IBA resistance in *pex7-2*

Because the energy for seedling establishment is dependent on peroxisomal fatty-acid β-oxidation, seedlings defective in peroxisomal function are also defective in post-germinative growth unless provided with exogenous energy sources such as sucrose. Although, *pex7-2* is only slightly sucrose dependent in the dark (Ramón and Bartel, 2010) and *pex13-1* is not notably sucrose dependent (Chapter 3), the *pex7-2 pex13-1* double mutant was dramatically sucrose dependence in dark (Figure 6.1A, B).

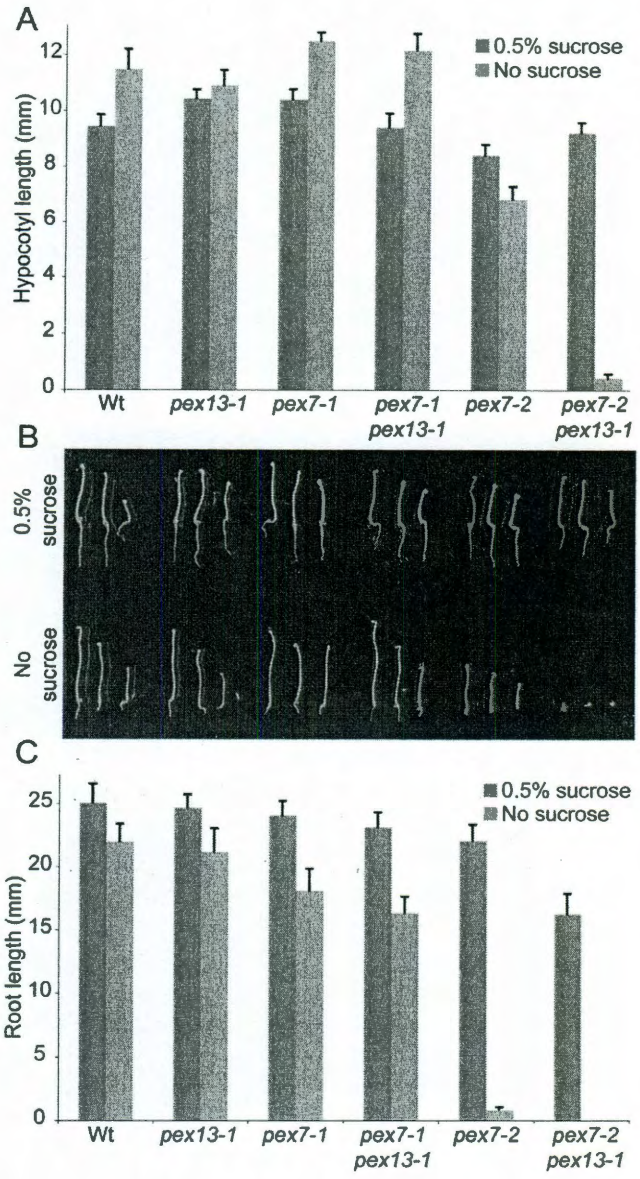

Figure 6.1 *pex13-1* differentially affects sucrose dependence in *pex7* alleles. (A) Mean hypocotyl lengths of 5-day old seedlings grown in the dark on medium with and without 0.5% sucrose. (B) Three seedlings representing a range of phenotypes for each line were photographed. Mean root lengths of 8-day-old seedlings grown in yellow light on medium with and without 0.5% sucrose are shown (C). Error bars in A and C represent standard error of the means ($n \geq 12$).

pex7-2 and *pex13-1* have more severe phenotypes when grown in the light. The *pex7-2* single mutant is already severely sucrose dependent in the light, and *pex13-1* enhanced *pex7-2* sucrose dependence (Figure 6.1C). Additionally, the double mutant had reduced root length even on media supplemented with sucrose (Figure 6.1C), indicating a more severe growth defect then either *pex7-2* or *pex13-1* single mutants.

Similar to fatty-acid β-oxidation, peroxisomes also house the enzymes for IBA to IAA conversion and 2,4-DB to 2,4-D conversion, and *pex* mutants are often resistant to the effects of IBA and 2,4-D metabolism, such as primary root inhibition and lateral root proliferation. *pex13-1* is not notably IBA resistant in either primary root elongation (Figure 6.2A) or lateral root inhibition assay (Figure 6.2D), and is not 2,4-DB resistant in primary root elongation (Figure 6.2C) whereas *pex7-2* is IBA resistant in all three assays (Ramón and Bartel, 2010). The *pex7-2 pex13-1* double mutant had enhanced IBA resistance in primary root elongation at low and high IBA concentrations (Figure 6.2A) and remained resistant to IBA-promoted lateral root proliferation (Figure 6.2D). Furthermore, *pex7-2 pex13-1* had enhanced resistance to 2,4-DB (Figure 6.2C). The enhancement of *pex7-2* sucrose dependence in the dark, IBA resistance, and 2,4-DB resistance by *pex13-1* is consistent with *pex13-1* double mutants made with early-acting *pex* mutants such as *pex14-2 pex13-1* (Chapter 4).

6.2 *pex13-1* enhances *pex7-2* PTS2 processing defects

To determine if the enhancement of the physiological phenotypes observed in the *pex7-2 pex13-1* double mutant was accompanied by enhanced molecular defects, I examined PEX levels and PTS2 import in the light and in the dark. *pex7-2* has reduced levels of PEX7 in the light and the dark (Ramón and Bartel, 2010) and this defect was

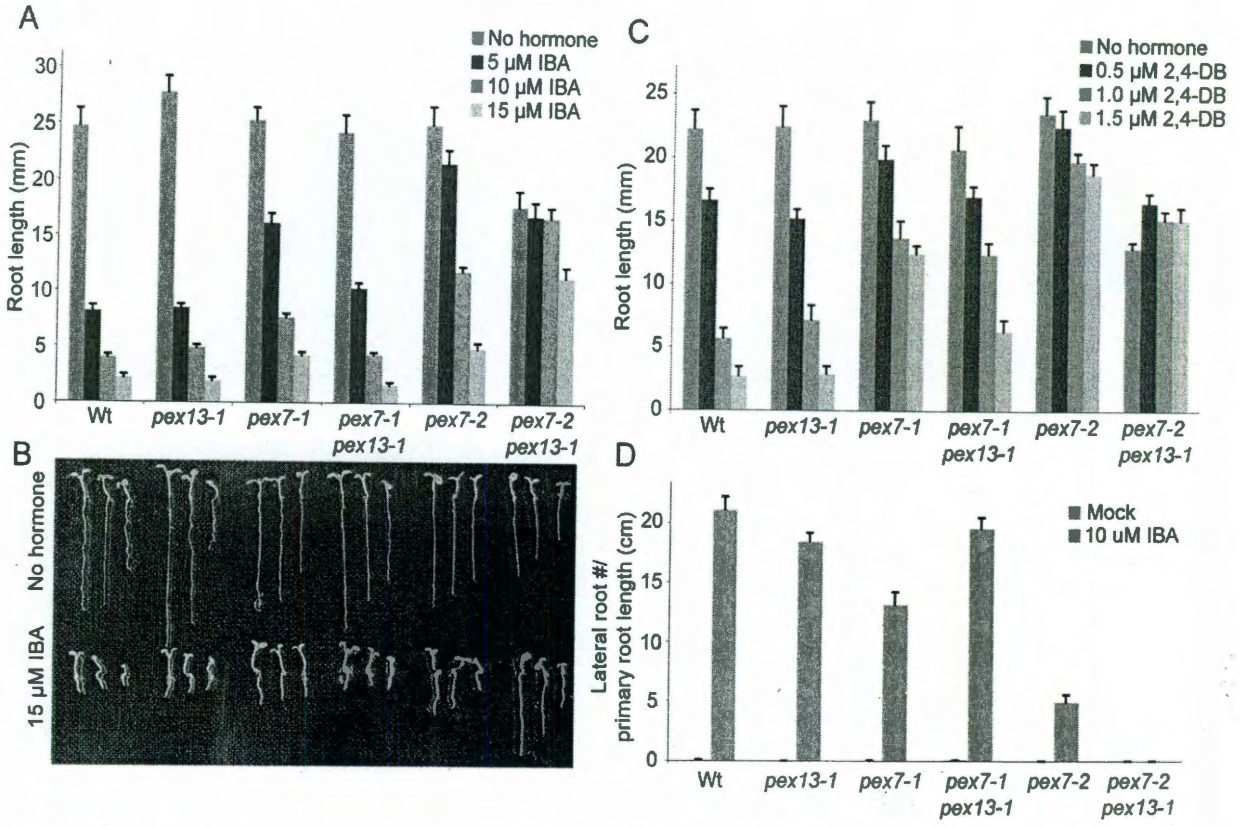

Figure 6.2 *pex13-1* differentially affects IBA and 2,4-DB resistance in *pex7* alleles. (A) Mean root lengths of 8-day-old seedlings grown under yellow light on 0.5% sucrose supplemented medium with and without the indicated concentration of IBA are shown. (B) Three representative seedlings from each line were photographed. (C) Mean root lengths of 8-day-old seedlings grown under yellow light on 0.5% sucrose supplemented medium with and without the indicated concentration of 2,4-DB are shown. (D) Four-day-old seedlings were transferred to medium with or without 10 μM IBA for an additional 4 days under yellow light, after which root lengths were measured and primary roots were counted. Error bars in panels A, C, and D represent standard error of the means ($n \geq 12$).

maintained in the *pex7-2 pex13-1* double mutant (Figure 6.3). PEX5 levels are also reduced in the *pex7-2* single mutant, but only when seedlings are grown in the light (Ramón and Bartel, 2010). Reduction of PEX5 levels was also seen in the *pex7-2 pex13-1* double mutant when grown in the light (Figure 6.3). PEX14 accumulated to wild-type levels in both *pex7-2* and *pex7-2 pex13-1* (Figure 6.3).

I also used immunoblotting to monitor the ratio of processed to mature PTS2 proteins, thiolase and PMDH, in *pex7-2 pex13-1*. *pex7-2* displayed a severe PTS2 processing defect in the light and the dark (Ramón and Bartel, 2010). PMDH processing in *pex7-2 pex13-1* double mutant was similar to the *pex7-2* single mutant (Figure 6.3). However, the thiolase processing defect was enhanced in the *pex7-2 pex13-1* double mutant in the light and the dark (Figure 6.3). The enhanced processing defect, IBA resistance, and sucrose dependence is consistent with a double mutant made with two single mutants in the same process and is consistent with both mutants being disrupted in an early-acting peroxin.

6.3 *pex13-1* suppresses *pex7-1* IBA resistance

To further characterize the genetic interaction between *PEX7* and *PEX13*, I examined the *pex7-1 pex13-1* double mutant. Unlike *pex7-2*, which has strong *pex* phenotypes and is a missense mutant (Ramón and Bartel, 2010), *pex7-1* is phenotypically less severe and is a T-DNA insertion mutant in the 5' UTR (Woodward and Bartel, 2005b). Like *pex13-1*, *pex7-1* has reduced *PEX7* mRNA levels, but it is likely that any protein that is expressed is functional.

To examine peroxisomal function in the *pex7-1 pex13-1* double mutant, I assayed sucrose dependence in the light and in the dark. Neither *pex7-1* nor *pex13-1* is notably

sucrose dependent and the double mutant also was not sucrose dependent in the light or in the dark (Figure 6.1).

To monitor slight alterations in IBA and 2,4-DB response, I measured IBA or 2,4-DB resistance over a range of concentrations. Although *pex13-1* was not IBA resistant at any tested IBA concentration, *pex7-1* was most clearly IBA resistant at low concentrations such as 5 and 10 μM IBA. The *pex7-1 pex13-1* double mutant was less resistant to IBA than the *pex7-1* single mutant at 5 μM IBA (Figure 6.2C). IBA responses of *pex7-1 pex13-1* were similar to wild type at higher concentrations. *pex13-1* is not 2,4-DB resistant and *pex7-1* is 2,4-DB resistant at all concentrations tested (Figure 6.2C). The *pex13-1* lesion partially suppressed *pex7-1* 2,4-DB resistance in the double mutant. These results suggested that the defect in *pex7-1* could be rescued in a similar manner to late-acting mutants and were in contradiction to the conclusions from the *pex7-2 pex13-1* analysis (Section 6.1-2).

6.4 *pex7-1 pex13-1* has restored PEX7 levels, PEX5 levels, and PTS2 processing

To determine if the suppression of the physiological phenotypes was associated with a suppression of the molecular phenotypes, I monitered PEX levels via immunobloting. *pex7-1* has reduced accumulation of PEX7 and PEX5 and normal accumulation of PEX14 (Figure 6.3, Ramón and Bartel, 2010). PEX14 accumulated to wild-type levels in *pex7-1 pex13-1* (Figure 6.3). Surprisingly, however, the double mutant had restored levels of both PEX7 and PEX5 (Figure 6.3). The restoration of PEX7 and PEX5 receptor levels in *pex7-1 pex13-1* was accompanied by rescued thiolase and PMDH processing (Figure 6.3). The molecular analysis of *pex7-1 pex13-1* suggests

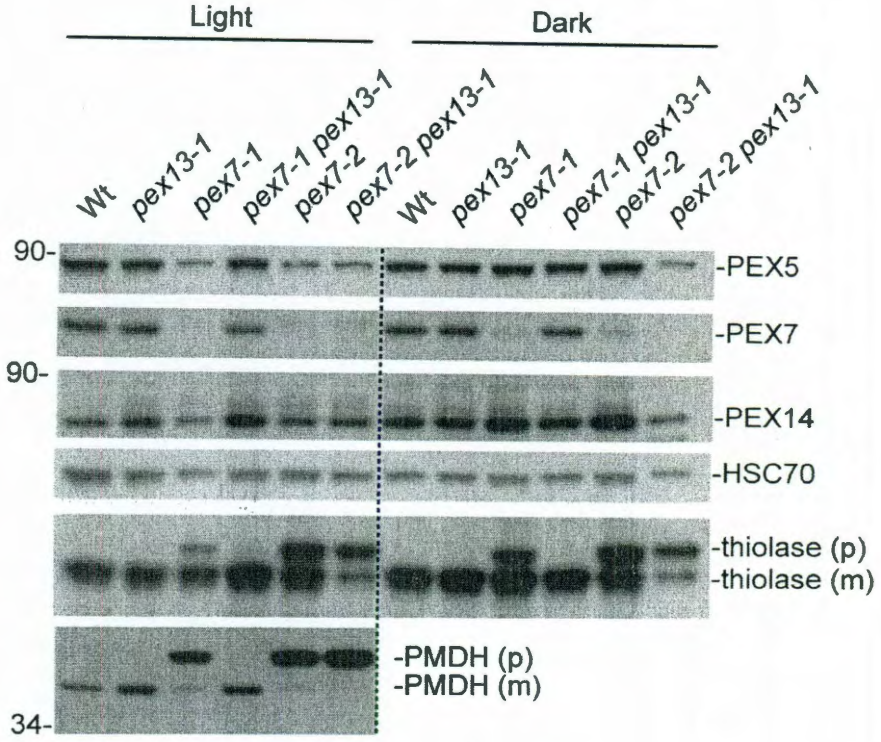

Figure 6.3 *pex13-1* suppresses *pex7-1* but does not suppress *pex7-2*.
Protein extracts from eight 5-day-old seedlings grown in the light or in the dark on medium with 0.5% sucrose were processed from immunoblotting using the indicated antibodies. Precursor (p) and mature (m) proteins contain or lack, respectively, the N-terminal PTS2 peptide. α-HSC70 was used as a loading control. Positions of molecular mass markers (in kDa) are indicated on the left.

that PEX7 may have a role receptor ubiquitination or recycling similar to other late-acting mutants that are suppressed by *pex13-1* (Chapter 5). However, in both the molecular and physiological assays, the rescue of *pex7-1 pex13-1* contradicts the enhancement of *pex7-2 pex13-1*.

6.5 Conclusions

I found that early-acting mutants were enhanced when combined with *pex13-1* (Chapter 4) and late-acting mutants were suppressed when combined with *pex13-1* (Chapter 5). To explore the role of PEX7 in peroxisomal import and receptor recycling, I isolated and characterized *pex7 pex13-1* double mutants.

Because the most well defined role for PEX7 is as a peroxisomal receptor, I expected that *pex7 pex13-1* would respond to these assays as an early-acting *pex* mutant would. I was surprised to find opposing results between *pex7-1 pex13-1* and *pex7-2 pex13-1* double mutants. Similar to the *pex13-1* enhancement of early-acting *pex* mutants (Chapter 4), I found that *pex13-1* enhanced the sucrose dependence (Figure 6.1), IBA and 2,4-DB resistance (Figure 6.2), and PTS2 processing defects of *pex7-2*.

Similar to the *pex13-1* suppression of late-acting mutants (Chapter 5), I found that *pex13-1* fully restored *pex7-1* sucrose dependence (Figure 6.1), IBA and 2,4-DB resistance (Figure 6.2), reduced PEX5 and PEX7 levels (Figure 6.3), and PTS2 processing defects (Figure 6.3). At first glance, these results suggest that in addition to being a peroxisomal receptor, PEX7 may have an additional role; perhaps in PEX5 release from the peroxisomal pore or in recycling. This explanation would be consistent with the rescue of PEX5 levels in the *pex7-1 pex13-1* double mutant (Figure 6.3). The rescue of PEX7 levels, however, is more difficult to understand.

There are several possibilities that could explain the restored accumulation of PEX7 in the *pex7-1 pex13-1* double mutant. The most obvious possibility, and the one I ruled out first, is that the line is not homozygous for the *pex7-1* mutation. After genotyping in multiple generations, and repeating the phenotypic assays and immuoblotting, I confirmed that the lines were homozygous for the *pex7-1* mutation and that the accumulation of PEX7 was reproducible. The second possibility is that reduced PEX13 levels stabilize PEX7 protein. However, I did not see increased levels of PEX7 in other *pex13-1* double mutants (Figure 4.2 and 5.3), including *pex7-2 pex13-1* (Figure 6.3), or in the *pex13-1* (Chapter 3) or *pex13-4* single mutants (Figure 3.3).

There is another convoluted, yet possible, explanation that must also be ruled out. *pex7-1* may have reduced levels of PEX7 due to T-DNA silencing of the *PEX7* mRNA. When the *pex13-1* lesion is added, this silencing may be released, allowing PEX7 levels to accumulate to wild type levels. To distinguish between the possibilities that PEX7 has an additional role in peroxisomal function or that the rescue I observed was due to a restoration of *PEX7* mRNA levels, RNA gel blot analysis would be informative. In addition, I am isolating *pex7 pex13-4* double mutants. Preliminary data suggests that *pex13-4* functions analogously to *pex13-1* in the early- and late-acting assays with *pex4-1* (data not shown). Because *pex13-4* is not a T-DNA allele, combining the *pex7-1* and *pex13-4* should not relieve any hypothetical T-DNA silencing of the *pex7-1* insertion.

Chapter 7: *pex4-1* Defects are Related to PEX5 Overaccumulation in the Peroxisome

Certain late-acting *pex* mutants have reduced accumulation of PEX5. For example, in yeast, mammals, and plants, *pex6* mutants have dramatically reduced PEX5 (Dodt and Gould, 1996; Collins et al., 2000; Zolman and Bartel, 2004). However, PEX5 levels in *pex4* mutants vary by species (Koller et al., 1999; Collins et al., 2000; Kiel et al., 2005a). In Arabidopsis, *pex4-1* mutants have normal PEX5 levels (Zolman et al., 2005); however, the available PEX5 accumulates in the peroxisome (Chapter 5, Ratzel et al., 2010). To evaluate the consequences of peroxisomal PEX5 accumulation on peroxisome function, I characterized the physiological phenotypes of the late-acting mutant *pex4-1* overexpressing *PEX5* or *PEX7* compared to the previously described *pex6-1* overexpressing *PEX5*. Furthermore, I investigated the localization of the receptors PEX5 and PEX7 and the peroxisomal matrix proteins in *pex4-1* overexpressing *PEX5* or *PEX7* and *pex6-1* overexpressing *PEX5*. I found that the paradoxical *pex4-1* phenotypes can be reconciled with the hypothesis that in *pex4-1*, PEX5 peroxisomal pores may be stabilized allowing either loss of required metabolites or diffusion of toxic byproducts into the cytosol.

7.1 *pex4-1* sucrose dependence is enhanced by *PEX5* overaccumulation

To elucidate the molecular basis for the physiological defects resulting from reduced receptor recycling, I compared the effects of overexpressing the PTS1 receptor *PEX5* from the strong cauliflower mosaic virus 35S promoter in *pex4-1* and *pex6-1* plants. Before photosynthesis is established, oilseed plants such as *Arabidopsis* rely on

β-oxidation of stored fatty acids for energy. Because peroxisomes are the sole site of fatty acid β-oxidation in plants (Baker et al., 2006), *pex* mutants typically display growth defects, such as reduced hypocotyl and root length, that can be at least partially rescued by an exogenous fixed carbon source, such as sucrose (Hayashi et al., 1998; Zolman et al., 2000). *pex4-1* and *pex6-1* are missense alleles that are sucrose dependent in the dark and in the light (Zolman and Bartel, 2004; Zolman et al., 2005; Ratzel et al., 2010), and overexpressing *PEX5* partially restores sucrose independence to dark-grown *pex6-1* (Zolman and Bartel, 2004).

To compare the effects of overexpressing *PEX5* on *pex4-1* and *pex6-1* sucrose dependence, I grew plants on medium supplemented with and without sucrose in the dark and in the light. Wild-type seedling roots and hypocotyls elongate similarly on medium with and without sucrose. As previously reported (Zolman and Bartel, 2004; Zolman et al., 2005; Ratzel et al., 2010), light-grown roots and dark-grown hypocotyls of *pex4-1* and *pex6-1* mutants are considerably shorter than wild type in the absence of sucrose supplementation (Figure 7.1A, 1B). Overexpressing *PEX5* partially rescued the sucrose dependence of *pex6-1* root elongation in the light (Figure 7.1A) and hypocotyl elongation in the dark (Figure 7.1B). In contrast, overexpressing *PEX5* enhanced the sucrose dependence of *pex4-1* root elongation in the light (Figure 7.1A) and slightly enhanced the sucrose dependence of *pex4-1* hypocotyl elongation in the dark (Figure 7.1B). To determine if this enhancement was specific to the PEX5 receptor, I assayed *pex4-1* overexpressing the PTS2 receptor *PEX7*, and no alteration of sucrose dependence was observed in the light (Figure 7.1A) or the dark (Figure 7.1B).

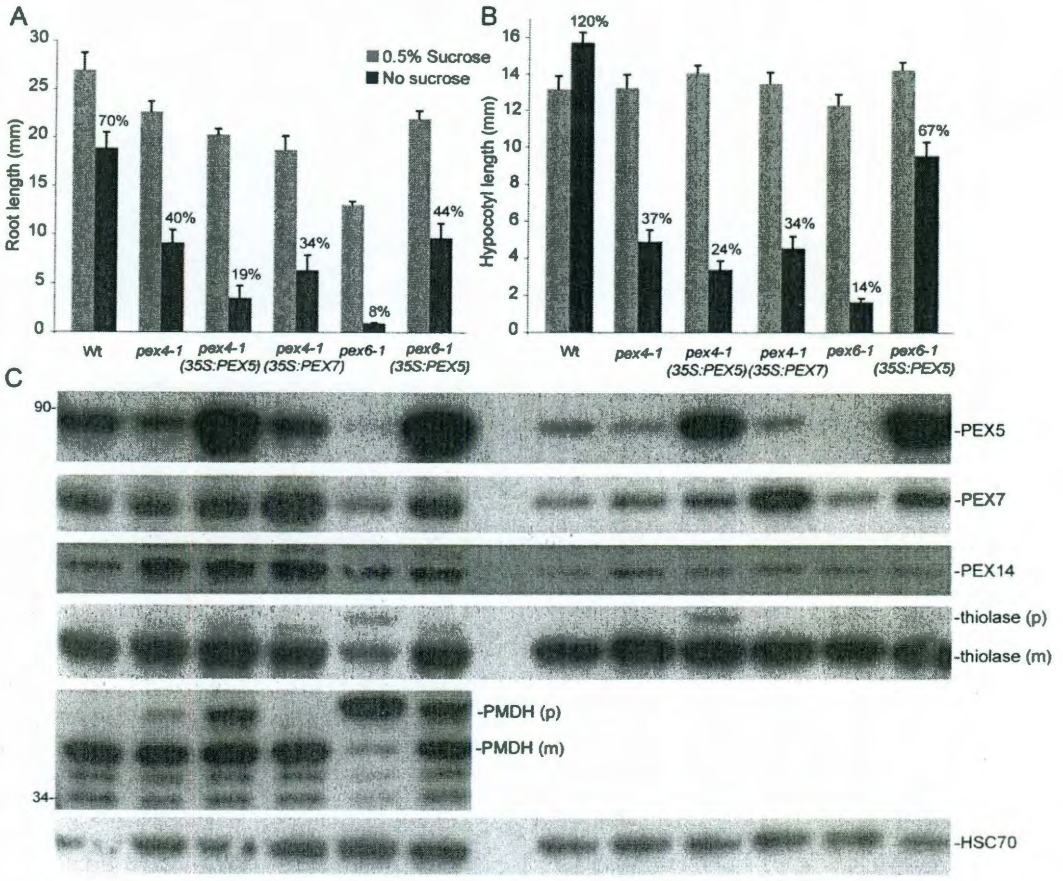

Figure 7.1 *PEX5* overexpression enhances *pex4-1* sucrose dependence and PTS2 processing defects but suppresses *pex6-1* sucrose dependence and PTS2 processing defects.

A. Mean root lengths of 8-day-old seedlings grown in the light on medium with and without 0.5% sucrose. B. Mean hypocotyl lengths of 5-day-old seedlings grown in the dark on medium with and without 0.5% sucrose. Percentages above the bars represent the percent elongation on medium lacking sucrose compared to growth on sucrose-supplemented medium. Error bars represent standard error of the means ($n \geq 12$). C. Protein extracts from eight seedlings from sucrose dependence assay in the light (A) or dark (B) grown on medium with 0.5% sucrose were processed for immunoblotting and probed sequentially with the indicated antibodies. Precursor (p) and mature (m) proteins contain or lack, respectively, the N-terminal PTS2 peptide. α-HSC70 was used as a loading control, and the positions of molecular mass markers (in kDa) are indicated on the left.

Because driving cDNAs from the 35S promoter can cause either overexpression of the target or co-suppression of the target and endogenous mRNAs, we monitored PEX5 and PEX7 levels in the transformed *pex4-1* and *pex6-1* lines using immunoblot analysis. As previously reported (Zolman and Bartel, 2004), *pex6-1* seedlings displayed reduced PEX5 levels (Figure 7.1C). We found that both *pex4-1* (*35S:PEX5*) and *pex6-1* (*35S:PEX5*) accumulated more PEX5 protein than either wild type or the untransformed mutants, and that PEX7 levels were elevated in *pex4-1 (35S:PEX7)* (Figure 7.1C). Overexpression of *PEX5* in *pex4-1* or *pex6-1* did not appear to alter PEX7 or PEX14 levels, and overexpression of *PEX7* in *pex4-1* did not appear to alter PEX5 or PEX14 levels (Figure 7.1C). I concluded that the enhanced sucrose dependence observed following introduction of the *35S:PEX5* construct into *pex4-1* was likely caused by PEX5 over-accumulation rather than by *PEX5* co-suppression.

7.2 *pex4-1* IBA resistance is enhanced by PEX5 overexpression

Genetic and biochemical evidence suggests that IBA-to-IAA conversion occurs in peroxisomes in a manner similar to fatty acid β-oxidation (Zolman et al., 2000; Zolman et al., 2007; Zolman et al., 2008; Strader et al., 2010). As a result, many *pex* mutants, including *pex4-1* and *pex6-1*, are defective in IBA responses such as IBA-mediated primary root inhibition and IBA promotion of lateral roots (Zolman and Bartel, 2004; Zolman et al., 2005). Consistent with the enhanced sucrose dependence (Figure 7.1A, B), I found that PEX5 overaccumulation slightly enhanced *pex4-1* resistance to a range of IBA concentrations in primary root elongation (Figure 7.2A), whereas PEX7 overaccumulation did not appreciably alter *pex4-1* IBA resistance. *pex6-1* (*35S:PEX5*) lines remain IBA resistant in root elongation, although *PEX5* overexpression does restore

the root elongation defect displayed by *pex6-1* even when sucrose supplemented (Zolman and Bartel, 2004). Consistent with the suppressed sucrose dependence (Figure 7.1A, B), I found a slight restoration of IBA responsive root elongation inhibition in *pex6-1* overexpressing *PEX5* (Figure 7.2A).

A second functional assay of peroxisomal IBA β-oxidation is the promotion of lateral roots by exogenous IBA. In wild type, lateral root initiation can be promoted by IBA or napthaleneacetic acid (NAA), a synthetic auxin that does not require β-oxidation for activity (Figure 7.2B). *pex4-1*, *pex4-1* (*35S:PEX5*), and *pex4-1* (*35S:PEX7*) were all similarly resistant to the promotive effect of IBA on lateral root production. As in the root elongation assay, overexpression of *PEX5* slightly restored IBA responsive lateral root production in *pex6-1*, although IBA responsiveness did not return to wild-type levels (Figure 7.2B). All mutants initiated lateral roots in response to 80 nM NAA similarly to wild type, implying that the IBA resistance was not due to the inability to develop lateral roots.

7.3 Overaccumulation of PEX5 enhances PTS2 processing defects in *pex4-1*

To determine if the enhanced physiological phenotypes of *pex4-1* (*35S:PEX5*) were accompanied by alterations in matrix protein import, I monitored PTS2 processing using immunoblot analysis. PTS2 proteins are cleaved of the target signal by a PTS1 enzyme, DEG15, upon entrance into the peroxisome (Helm et al., 2007; Schumann et al., 2008). Therefore, efficient PTS2 processing requires both PTS1 and PTS2 import, and PTS2 processing defects can reflect defects in PTS1, PTS2, or a combination of PTS1 and PTS2 peroxisomal import.

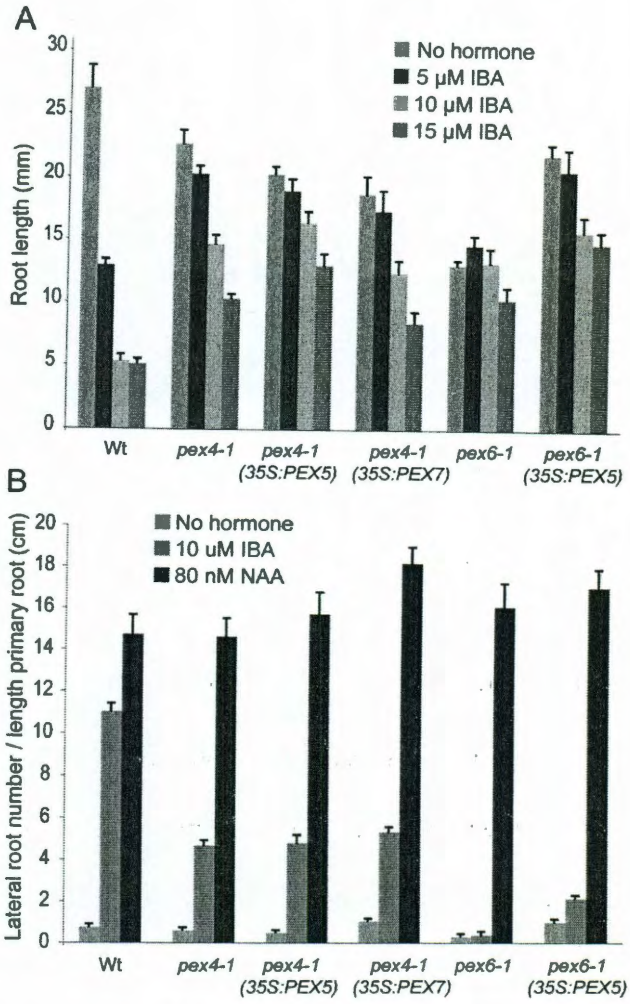

Figure 7.2 *PEX5* overexpression enhances *pex4-1* but not *pex6-1* IBA resistance. A. Mean root lengths of 8-day-old seedlings grown under yellow-filtered light on medium supplemented with 0.5% sucrose and the indicated concentration of IBA. B. Four-day-old seedlings were transferred to medium with or without 10 µM IBA or 80 nM NAA for an additional 4 days under yellow-filtered light, after which root lengths were measured and primary roots were counted. Error bars represent standard errors of the means ($n \geq 12$).

I monitored processing of two PTS2 proteins, 3-ketoacyl-CoA thiolase (thiolase) and peroxisomal malate dehydrogenase (PMDH), in seedlings from the control plates containing sucrose from the sucrose dependence assays (Figure 7.1C). Thiolase and PMDH are fully processed in wild-type seedlings, whereas *pex4-1* and *pex6-1* seedlings display partial PTS2 processing defects (Zolman and Bartel, 2004; Ratzel et al., 2010). I found that PEX5 overaccumulation enhanced the slight PMDH processing defect observed in 8-d-old light-grown *pex4-1* seedlings, and resulted in a visible thiolase-processing defect in 5-d-old dark-grown *pex4-1* seedlings (Figure 7.1C). In contrast, PEX7 overaccumulation rescued the slight PMDH processing defect of *pex4-1* (Figure 7.1C). Consistent with the physiological rescue seen in the *pex6-1* (*35S:PEX5*) line (Figure 7.1A, 7.1B, 7.2), PTS2 processing of both thiolase and PMDH in 8-d-old light-grown *pex6-1* seedlings was improved by overexpressing *PEX5*. These PTS2 processing alterations suggested that the opposite physiological consequences of overexpressing *PEX5* in *pex4-1* and *pex6-1* might result from enhanced or reduced matrix protein import defects, respectively, in these mutants.

7.4 Overexpressing *PEX5* in *pex4-1* does not enhance peroxisomal matrix protein import defects

Although PEX5 levels are not reduced in *pex4-1*, whole seedling immunoblot analysis does not reveal the distribution of PEX5 between the cytosol and the peroxisome. To monitor PEX5 localization, I used centrifugation to fractionate extracts of wild type, *pex4-1*, and *pex4-1* (*35S:PEX5*) seedlings into cytosolic and organellar fractions and monitored the resulting fractions by immunoblotting (Figure 7.3A). I probed immunoblots with antibodies recognizing HSC70, which was found in both the

supernatant (S) fraction and the organellar pellet (P), and the mitochondrial complex V α subunit (mito ATPase), which was exclusively in the pellet, as expected for an organellar integral membrane protein (Figure 7.3A). As previously observed for PEX5 (Ratzel et al., 2010), both PEX5 and PEX7 were found in both the supernatant (S) fraction and the organellar pellet (P) in wild type, consistent with models in which PEX5 and PEX7 cycle in and out of the peroxisome as they deliver cargo. I also monitored two peroxisomal matrix proteins, and found both the PTS2 protein PMDH2 and the PTS1 protein malate synthase (MLS) enriched in the organellar fraction relative to HSC70 (Figure 7.3A).

When I performed this fractionation on extracts from *pex4-1* seedlings, the only notable defect we observed was a shift in the distribution of PEX5 to the organellar fraction (P) compared to the wild-type distribution (Figure 3A, Ratzel et al., 2010). The ratios of cytosolic to peroxisomal PEX7, MLS, and PMDH were similar in wild type and *pex4-1* (Figure 7.3A), consistent with previous reports that *pex4-1* does not display dramatic matrix protein import defects (Zolman et al., 2005; Ratzel et al., 2010). As previously reported (Chapter 5, Ratzel et al., 2010), unprocessed PMDH was largely associated with the organellar pellet in *pex4-1*, suggesting either that the DEG15 protease was less abundant or less active in *pex4-1* peroxisomes.

The enhanced physiological defects observed in *pex4-1* (*35S:PEX5*) were accompanied by reduced PMDH processing but were not associated with notable mislocalization of MLS or PMDH to the cytosol, which would be indicated by an increased ratio of cytosolic to peroxisomal localization compared to *pex4-1*. Overall PEX5 levels were higher in total homogenate and cytosolic fractions in *pex4-1* (*35S:PEX5*), and the ratio of cytosolic to peroxisomal PEX7 was similar to wild type

(Figure 7.3A). The results from *pex4-1 (35S:PEX5)* fractionation experiments were consistent with the idea that PEX5 overaccumulation enhanced *pex4-1* physiological phenotypes through a mechanism independent of impeding matrix protein import.

7.5 Overexpressing *PEX5* in *pex6-1* partially restores peroxisomal matrix protein import

To understand the molecular nature of the *pex6-1* rescue by *PEX5* overexpression, I similarly fractionated extracts from wild type, *pex6-1*, and *pex6-1* (*35S:PEX5*) seedlings. PEX5 levels are reduced in *pex6-1* (Zolman and Bartel, 2004; Ratzel et al., 2010), and the remaining PEX5 is largely localized to the peroxisome (Chapter 5, Ratzel et al., 2010), as reflected in the shift of PEX5 from the cytosol to the peroxisomal fraction (Figure 7.3B). Reduced levels of MLS and PMDH were found in the organellar fraction compared to the supernatant, and substantial unprocessed PMDH was found in the cytosol, suggesting that *pex6-1* is impaired in both PTS1 and PTS2 import pathways (Chapter 5, Ratzel et al., 2010). Overexpressing *PEX5* in *pex6-1* resulted in a PEX5 distribution between the cytosol and peroxisome that was more similar to wild type (Figure 7.3B). This restoration was accompanied by improved MLS and PMDH peroxisomal localization compared to *pex6-1*. The results of these fractionation experiments were consistent with the possibility that *PEX5* overexpression restores *pex6-1* physiological phenotypes by improving matrix protein import.

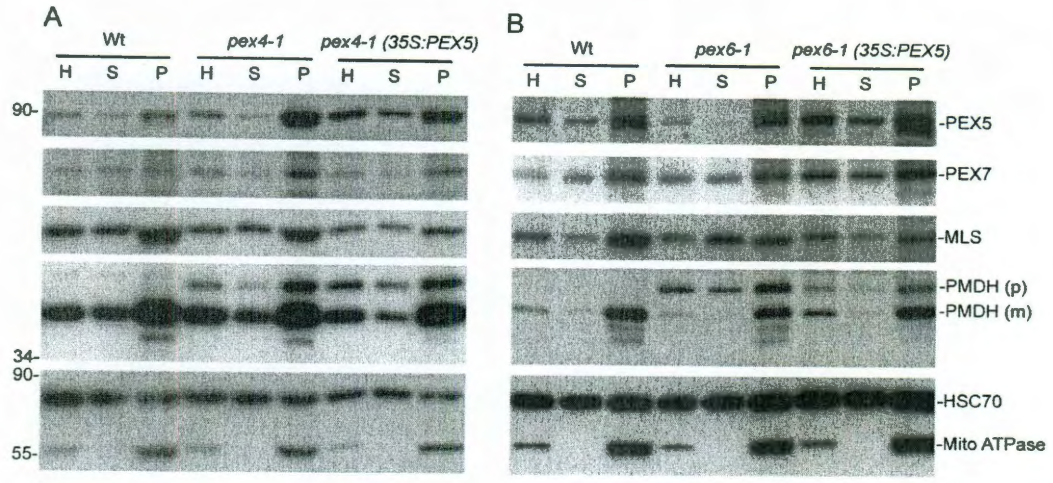

Figure 7.3 *PEX5* overexpression does not markedly alter *pex4-1* peroxisomal import but partially restores *pex6-1* peroxisomal import defects.
Whole seedling homogenates from 5-day-old light-grown seedlings of the indicated genotypes were separated by centrifugation into soluble and organellar pellet fractions. For each sample, 1% of the total homogenate (H), 1% of the soluble fraction (S), and 25% of the pellet fraction (P) were separated using SDS-PAGE and processed for sequential immunoblotting using the indicated antibodies. The mitochondrial membrane complex V subunit α (mito ATPase) and cytosolic HSC70 were used as organellar and cytosolic controls, respectively. Precursor (p) and mature (m) proteins contain or lack, respectively, the N-terminal PTS2 peptide.

7.6 Conclusions

Because the energy required for growth before photosynthesis is provided by peroxisomal fatty-acid β-oxidation, and because IBA is similarly β-oxidized to IAA in the peroxisome, Arabidopsis *pex* mutants, such as *pex6-1*, often are sucrose dependent and IBA resistant (Zolman and Bartel, 2004). Like yeast (Collins et al., 2000) and mammalian *pex6* mutants (Dodt and Gould, 1996), Arabidopsis *pex6-1* has reduced PEX5 levels (Zolman and Bartel, 2004), presumably because PEX5 is degraded in the absence of efficient retrotranslocation. The observation that overexpressing *PEX5* rescues *pex6-1* sucrose dependence (Figure 7.1-3, Zolman and Bartel, 2004) and slightly restores *pex6-1* IBA responsiveness (Figure 7.2) suggests that the decreased PEX5 levels in *pex6-1* limits peroxisomal matrix protein import. Indeed, I found that *pex6-1* matrix protein import defects, detected by reduced PTS2 processing (Figure 7.1C) and by association of PTS1 and PTS2 proteins with the organellar pellet upon centrifugal fractionation (Figure 7.3B), were restored by *PEX5* overexpression.

Both the physiological and molecular evidence support the hypothesis that reduced PEX5 levels and consequent reductions matrix protein import contribute to *pex6-1* phenotypes. However, because the rescue of *pex6-1* matrix protein import and physiological defects are not completely rescued by overexpressing *PEX5*, there are likely other contributing factors.

Like *pex6-1*, *pex4-1* is defective in a late-acting peroxin and is sucrose dependent and IBA resistant (Zolman et al., 2005). Unlike the rescue of *pex6-1* by overexpressing *PEX5*, however, we found that overexpressing *PEX5* enhanced the sucrose dependence (Figure 7.1A), IBA resistance (Figure 7.2), and PTS2 processing defects (Figure 7.1C) of

pex4-1. These results not only suggest that *pex4-1* phenotypes are not caused by reduced PEX5 function, but also indicate that excess PEX5 is deleterious to *pex4-1* peroxisome function. In contrast, overexpressing *PEX7* in the *pex4-1* mutant had no effect on either sucrose dependence or IBA resistance (Figure 7.1 and 7.2), suggesting that the enhancement seen in the *pex4-1* (*35S:PEX5*) line was specific to PEX5 overaccumulation and not generalizable to receptor overexpression.

In contrast to *pex6-1*, and in spite of severe peroxisome-defective phenotypes, neither *pex4-1* nor *pex4-1* (*35S:PEX5*) showed marked matrix protein import defects (Figure 7.3A). One notable molecular defect that is seen in *pex4-1* is an accumulation of PEX5 in the peroxisomal fraction (Figure 7.3A, Ratzel et al., 2010). Together, these results suggest that *pex4-1* physiological defects might result not from decreased matrix protein import, but rather from a toxic effect of accumulating PEX5 in the peroxisome. As PEX5 is proposed to form a pore through which matrix proteins enter the peroxisomes (Meinecke et al., 2010), I hypothesize that failure to efficiently remove PEX5 might result in leakage of peroxisome contents, either resulting in loss of substrates or cofactors needed for the metabolic reactions sequestered in the peroxisomal matrix, or leaching of toxic byproducts of peroxisomal reactions such as hydrogen peroxide.

To distinguish between the possibilities of reduced peroxisomal activity due to metabolite loss or toxicity due to hydrogen peroxide leakage, it would be interesting to measure hydrogen peroxide levels in wild type, *pex4-1*, *pex4-1 (35S:PEX5)*, and *pxa1*, the peroxisomal transporter that brings fatty acids and IBA into the peroxisome. Furthermore, leakiness could be measured using measured diffusion rates of a peroxisomal dye or acetyl-CoA (discussed in Section 8.2).

Chapter 8: Conclusions and Future Directions

8.1 Balance in peroxisomal import and receptor recycling

In this work, I used double mutant analysis to investigate the role of balance between peroxisomal import and receptor recycling. Using new *pex13* alleles (Chapter 3), I characterized the differential effects of reduced *PEX13* function on early-acting (Chapter 4) and late-acting (Chapter 5) peroxisomal mutants. Because double mutants harboring two lesions in import (e.g., *pex5-1 pex13-1* or *pex5-1 pex7-1*) or in recycling (e.g., *pex4-1 pex6-1*) enhance one another, whereas mutants that are symmetrical (e.g., *pex4-1 pex13-1* or *pex6-1 pex13-1*) suppress one another, I proposed the balance model (Chapter 5).

The balance model proposes that symmetrical disruptions (in early- and late-acting peroxins) suppress one another because of rebalanced peroxisomal import and receptor recycling. For example, *pex6-1* has reduced PEX5 accumulation (Zolman and Bartel, 2004) probably due to RADAR. Because import and recycling are cyclical, reduced PEX5 results in reduced import in *pex6-1* (Chapter 5 and 7, Ratzel et al., 2010). In Chapters 5 and 7, I described two ways in which PEX5 balance can be re-established in the *pex6-1* background. The first way to balance import and recycling would be to make a double mutant with *pex13-1*, a mutant that might result in reduced receptor import (Ratzel et al., 2010). When *pex6-1* and *pex13-1* are combined, *pex13-1* may slow the amount of receptor entering the peroxisome and subsequently degradation by *pex6-1*. Conversely, overexpressing PEX5 also rescues *pex6-1* (Chapter 7, Zolman and Bartel, 2004). This rescue might be the result of increasing the amount of PEX5 to keep pace with the continual degradation of PEX5 in *pex6-1*.

Because the double mutants made with *pex13-1* allowed me to divide early- and late-acting mutants based on enhanced or suppressed phenotypes, respectively, I applied this system to the less characterized PTS2 receptor PEX7 (Chapter 6). As expected, *pex7-2 pex13-1* was enhanced as compared to either single mutant, but surprisingly, *pex7-1 pex13-1* was rescued (Chapter 6). Other evidence also places PEX7 in both the early- and late-acting mutant classes and has distinct differences from PEX5. Similar to early-acting mutants, PEX7 has an early-acting role as the PTS2 receptor (Woodward and Bartel, 2005b; Ramón and Bartel, 2010), and *pex5-1 pex7-1* is enhanced compared to either single mutant (Woodward and Bartel, 2005b). Similar to late-acting mutants, PEX7 has reduced levels of PEX5 (Ramón and Bartel, 2010), and *pex4-1 pex7-1* has enhanced sucrose dependence and IBA resistance (data not shown). Future work will be required to determine if there is a role for PEX7 in the late-acting steps (discussed in Section 8.3).

8.2 New insights into *pex4-1* defects

I also found that localization of the PEX5 receptor could contribute to an imbalance in peroxisomal function using the *pex4-1* mutant (Chapter 5 and 7). Paradoxically, *pex4-1* has severe physiological defects suggestive of malfunctioning peroxisomes, yet seems to import matrix proteins almost normally (Zolman et al., 2005; Ratzel et al., 2010). I found that PEX4 function is required for reduction of PEX5 levels in *pex6-1* (Chapter 5, Ratzel et al., 2010), and this result hinted that in Arabidopsis PEX4 might be required for both mono- and poly-ubiquitination of PEX5, a prerequisite for removal from the peroxisome. In contrast, yeast uses different ubiquitin conjugating enzymes for mono- and poly-ubiquitination of PEX5 (Kiel et al., 2005a; Kragt et al.,

2005). To examine PEX5 localization, I performed cellular fractionation, and found that *pex4-1* overaccumulates PEX5 in the organellar fraction (Chapter 5, Ratzel et al., 2010). Furthermore, *pex4-1* defects can be enhanced by overexpressing PEX5 (Chapter 7). To resolve the contradiction that *pex4-1* displays peroxisome-defective phenotypes but lacks dramatic import defects, I hypothesize that PEX5 may not be efficiently removed from the peroxisome, resulting stabilized peroxisomal pores (Chapter 7). Interestingly, *pex4-1* is almost completely rescued when combined with the *pex13-1* mutation (Chapter 5, Ratzel et al., 2010) and this rescue correlates with a redistribution of PEX5 back to the cytosol (Chapter 5, Ratzel et al., 2010).

Because sucrose dependence and IBA resistance defects are enhanced with increasing levels of peroxisomal PEX5 but not with enhanced import defects (Chapter 5 and 7), it is possible that these suggested pores are leaking peroxisomal contents into the cytosol. The nature of these peroxisomal contents is undetermined. Two attractive possibilities include, peroxisomal metabolite or cofactors, which would reduce β-oxidation capacity and peroxisomal function, or the leaking contents could be peroxisomal reaction byproducts like hydrogen peroxide, which would be toxic in the cytosol.

Future studies to compare peroxisomal leakage and hydrogen peroxide levels in wild type, *pex4-1*, and *pex4-1* overexpressing PEX5 may be informative. For example, time course studies using isolated organelles loaded with a florescent peroxisomal dye, such as 8-(4-Nitrophenyl)-BODIPY (Landrum et al., 2010), could be used to asses dye leakage rates in wild type, *pex4-1* and *pex4-1* overexpressing PEX5. As an alternative to BOIDIPY staining, acetyl-CoA leakage assays could also be informative.

In addition to reverse genetic approaches to understanding *pex4-1* interactions (Chapter 5 and 7), I performed a *pex4-1* suppressor screen (Appendix A). These suppressors may include mutants in early-acting peroxins and proteins required for PEX5 removal from the peroxisome. Identification of the genes disrupted in these lines could provide additional insight into receptor recycling.

8.3 *pex13-4* as a new tool to study PEX interactions

A new tool for dissecting PEX interactions will be *pex13-4*. My initial characterization revealed that it is a unique allele because other *pex13* alleles are either lethal (Boisson-Dernier et al., 2008) or weak (Mano et al., 2006; Ratzel et al., 2010). *pex13-4* has reduced PEX13 accumulation, severe sucrose dependence, and IBA resistance (Chapter 3) that may be a result of pex13-4 reduced affinity for PEX14. Characterization of *pex13-4* overexpressing PEX13 will be needed to confirm that the *pex13-4* lesion confers the observed phenotypes. Two potential avenues for future work with *pex13-4* can be envisioned.

To test the hypothesis that the *pex13-4* lesion disrupts PEX14 interactions, yeast-two hybrid analysis with various constructs of PEX13, PEX14, pex13-4, and truncated PEX13 fragments could be performed. My initial growth and β-galactosidase assays failed to verify that wild-type PEX13 could interact with PEX14 (data not shown). However, the interaction of PEX13-PEX14 in yeast is weak and possibly transient where the $K_d \approx 50$ nM (Pires et al., 2003). As an alternative approach, truncated fusion proteins of GST-PEX13 and His-PEX14 (from pGEX-4T-GST-PEX13(215-304) and pET28-6xHis-PEX14(1-242) that were generated in antibody production), could be purified to measure interactions using surface plasmon resonance.

To determine if *pex13-4* double mutants behave similarly to *pex13-1* double mutants, it would be interesting to characterize double mutants with *pex13-4*. My preliminary findings suggest that *pex4-1 pex13-4* is rescued (data not shown) and crosses between *pex13-4* and *pex5-1*, *pex5-10*, *pex7-1*, and *pex14-1* have been carried out. Crosses of *pex13-4* with *pex6-1* and *pex7-2* produced no seed but could be repeated. Moreover, the *pex7-1 pex13-4* double mutant could be compared to the *pex7-1 pex13-1* double mutant to determine if the nature of the rescue in the *pex7-1 pex13-1* double mutant (discussed in Chapter 6).

8.4 Overexpression of docking complex proteins: PEX13 and PEX14

Double mutants made with *pex13-1*, harboring reduced levels of *PEX13*, provided considerable insight into the relationship between early- and late-acting *pex* mutants. Conversely, lines overexpressing PEX13 might display reciprocal phenotypes as the *pex13-1* double mutants. Evidence from yeast, where overexpressing either PEX13 or PEX14 results in *pex* phenotypes but overexpressing both PEX13 and PEX14 results in normal growth (Bottger et al., 2000), points to the potential problem in overexpressing PEX13 in lines with wild-type levels of PEX13 and PEX14. Indeed, when I tried overexpressing PEX13 in wild type and *pex13-1*, I recovered lines that harbored Basta resistance, indicating presence of the overexpression vector, but did not overaccumulate PEX13, suggesting transgenic plants with the desired expression levels have not have been healthy enough to survive selection (data not shown).

To determine if PEX14 can be overexpressed independently of PEX13, lines overexpressing PEX14 would be informative. I have transformed *pex14-2*, which does not accumulate full length PEX14 (Monroe-Augustus et al., 2010), along with wild type,

pex13-1, *pex7-1*, and *pex7-2*, with wild type PEX14 driven by the 35S overexpression promoter. If this line overexpresses PEX14 and rescues *pex14-2* phenotypes, it will be interesting to learn the effects on wild type and various *pex* mutants.

To effectively increasing docking complexes in Arabidopsis, overexpression PEX13 and PEX14 may need to be simultaneous. If overexpression lines are recovered, future studies on the effect of overexpressing both PEX13 and PEX14 may provide insight into how the docking complex may contribute to the balance of peroxisomal import and recycling.

8.5 Chemical genomics screen: PEX13 inhibitors

In many ways, plant peroxisome biogenesis is similar to human peroxisome biogenesis. The same lesions that result in Arabidopsis *pex5-1* (Zolman et al., 2000) and *pex6-1* (Zolman and Bartel, 2004) mutants confer peroxisomal biogenesis disorders in mammals (Zhang et al., 1999; Matsumura et al., 2000). Because the majority of human peroxisomal disorders are a result of mutations in late-acting peroxins (Crane et al., 2005; Ebberink et al., 2010) and because decreased PEX13 activity may suppress late-acting mutants (Ratzel et al., 2010), a chemical genomics screen for inhibitors of PEX13 function may identify potential therapeutic agent for plants and humans.* Because chemical genomics screens in plants are relatively facile, compounds could be quickly identified using a sucrose dependence assay on dark-grown seedlings in 96-well plates, and successful compounds could be used in plant studies as well as sent up a increasingly rigorous series of assays for human therapeutics.

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* A provisional patent has been filed describing this line of research.

For example, a compound that disrupted PEX13-PEX14 interactions might appear similar to a *pex13-4* mutant (which seems to suppress *pex4-1*). The disrupted interaction could be characterized by the appropriate method identified in Section 8.1. Furthermore, this compound could be tested in human cell culture and in lines with late-acting mutations to determine if they could be potentially used to treat the symptoms associated with peroxisomal biogenesis disorders caused by late-acting mutations.

8.6 PEX6 Interactors

Perhaps the largest gap in the Arabidopsis PEX family is the lack of a PEX15 homolog. PEX15 and/or PEX26 homologs are not apparent in the Arabidopsis genome, but I expect that a tether is needed to anchor the PEX6/PEX1 complex to the peroxisome in Arabidopsis, as in other organisms, including yeast and mammals (Birschmann et al., 2003; Matsumoto et al., 2003). Previous approaches to identify such a tether have included bioinformatics and a yeast-two hybrid screen baited with PEX6 (data not shown). I have initiated a new approach to identify PEX15 using a TAP-tagged (Earley et al., 2006) PEX6 fusion protein expressed in wild type and pex6-1. In the future, these lines could be used to identify PEX6 interactors, which might include PEX6, PEX1, PEX15 and accessory proteins that assist in PEX6 function.

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Appendix

In the work that I described in Chapters 3-7, I used reverse genetic approaches to gain insight into mechanisms late-acting peroxin function. However, there are still many questions that remain regarding the role of PEX4 and PEX6 in peroxisomal biogenesis. For example, the presumed PEX6 peroxisomal anchor, PEX15, has still not been identified in Arabidopsis. To better understand the roles and interacting partners of PEX4 and PEX6, I used a reverse genetics approach by performing suppressor screens in the *pex4-1* and *pex6-1* backgrounds.

A. Screen for suppressors of *pex4-1*

I screened 175,000 EMS mutagenized *pex4-1* M2 seeds for sucrose independence in the dark (SR lines). From this pool, 247 M3 seedlings were moved to soil and made seed, and undergraduate Amy Liao determined that 149 lines were still sucrose independent when retested for sucrose dependence in the M3 generation. Because it was essential that the line contain the *pex4-1* lesion, Chaya Murali and Amy Liao genotyped each line for *pex4-1*. I ruled out an additional 38 lines because they did not contain the *pex4-1* lesion. Together, 111 lines were sucrose independent and contained the *pex4-1* lesion. I focused my mapping efforts on the lines that had the strongest phenotypes and made outcrosses to Landsberg (Ler). Chaya Murali performed the initial mapping in lines SR165 and SR155.

SR165 partially restores IBA sensitivity to *pex4-1* (Figure A.1B), indicating a partial suppression of the IBA β-oxidation defect of *pex4-1*. Additionally, SR165 restores complete sucrose independence in the dark to *pex4-1* (Figure A.1A). Because SR165 M_4 lines segregate at a 1:1 ratio of sucrose dependent to independent seedlings,

the restoration of the sucrose independence appears to be dominant, indicating that this suppressor might be heterozygous at SR165 (Figure A1.C).

Mapping plants were selected for elongated hypocotyls when grown on medium lacking sucrose and subsequently genotyped for *pex4-1*. Initial mapping data of 12 seedlings suggested that the mapping interval was at the bottom of chromosome 4. Because the restoration of sucrose dependence and partial IBA resistance is similar to the *pex4-1 pex13-1* double mutant, I suspected that SR165 could contain a lesion in a peroxisome-associated protein. One gene, *PXA1*, fit the criteria of being peroxisomae-associated and was located at the bottom of chromosome 4. However, *PXA1* has not been fully sequenced from the SR165 mutant.

PXA1 is a peroxisomal transporter that is thought to transport fatty acids and IBA into the peroxisome in Arabidopsis and *pxa1-1* is sucrose dependent and IBA resistant (Zolman et al., 2001b). Furthermore, *pxa1-1/PXA1* heterozygotes are IBA resistant but not sucrose dependent, probably due to haploinsufficency (Zolman et al., 2001b).

Interestingly, several similarities could be drawn between the SR suppressor, if it is a lesion in *PXA1*, and the *pex4-1 pex13-1* double mutants. Certain *pxa1-1* and *pex13-1* phenotypes are dominant due to haploinsufficency (Chapter 5, Zolman et al., 2001b; Ratzel et al., 2010). *pxa1-1* and *pex13-1* cause reduced peroxisomal function by hindering entry of peroxisomal substrates or peroxisomal enzymes, respectively.

If SR165 is a *pxa1* allele, this result could also be put into context with the possibility that *pex4-1* defects are caused, at least in part, by leaking of toxic peroxisomal contents through stabilized PEX5 pores (Chapter 7). Because PXA1 transports

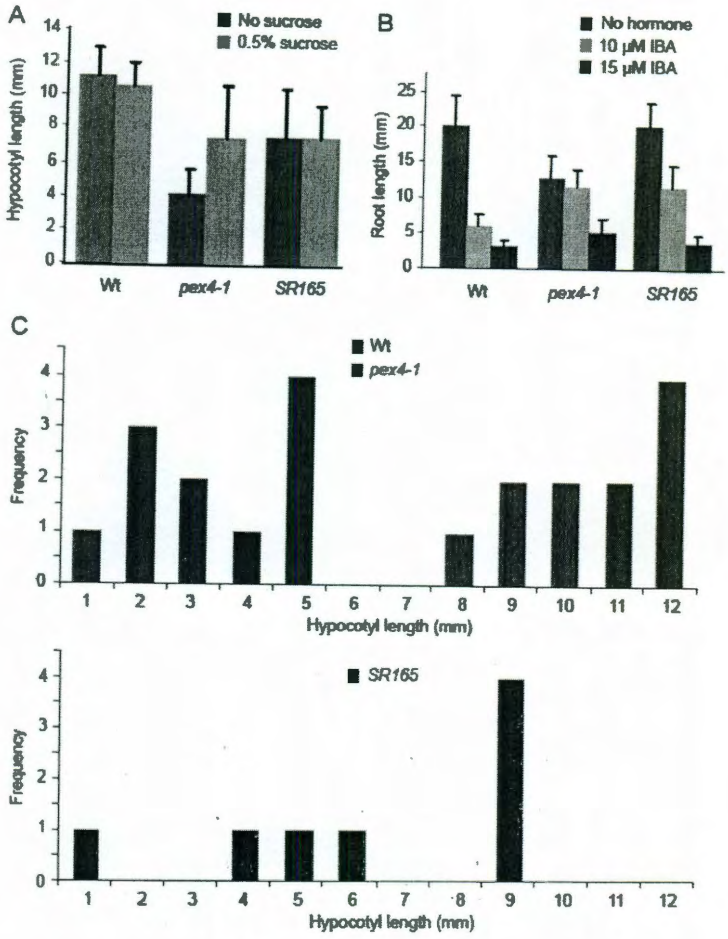

Figure A.1 SR165 is a dominant pex4-1 suppressor.
(A,B) SR165 suppresses *pex4-1* sucrose dependence but remains mildly IBA resistant. Mean hypocotyl length of 5-day-old seedlings grown in the dark on medium with and without 0.5% sucrose (A). Mean root lengths of 8-day-old seedlings grown under yellow light on medium 0.5% sucrose-supplemented medium with the indicated concentration of IBA (B). (C) Histogram of wild type, *pex4-1*, and SR165 M_4 segregating 5-day-old seedlings grown in the dark on medium without sucrose ($n \geq 8$). Error bars in panels A and B represent standard error of the means ($n \geq 10$).

peroxisomal substrates, *pxa1* mutant seedlings generate reduced hydrogen peroxide levels (Eastmond, 2007). Moreover, because the defect could be haploinsufficent, and because SR165 is heterozygous, perhaps transport is functional but at a reduced rate. This reduced import might allow for enough relief from the toxic leakiness for *pex4-1* to overcome sucrose dependence but remain mildly IBA resistant (Figure A1.A,B). In the future, it would be interesting to examine *pxa1 pex4-1* double mutants, and to identify the causative mutation in SR165.

B. Screen for suppressors of *pex6-1*

I characterized *pex6-1* modifiers isolated by Jeanne Rasberry from a screen of EMS mutagenized *pex6-1* seeds. Jeanne screened 27,000 M2 seeds for sucrose independence (named K lines) and 27,000 M2 seeds for enhanced IBA resistance (named Z lines) using the phenotypes of a long hypocotyl on medium lacking sucrose or long root on 15 μM IBA. Interestingly, screening for enhanced IBA resistance (Z modifiers) or for restored sucrose independence (K modifiers) yielded similar types of mutants; moderately restored sucrose independence with variable IBA resistance (Figures B.1, B.2). Because the Z and K modifiers do not fall into distinct phenotypic classes, we treated them as a single group of mutants.

pex6-1 is sucrose dependent and requires sucrose for full hypocotyl elongation (Zolman and Bartel, 2004). In contrast, wild-type hypocotyls elongate similarly on medium with and without sucrose. The *pex6-1* modifiers showed varied levels of restored sucrose independence, with Z35 being the most sucrose dependent followed by Z52, K276, Z39, K274, K284, Z188, Z40, and K287 being the least sucrose dependent (Figure B.1). The modifiers initially isolated for restored sucrose independence

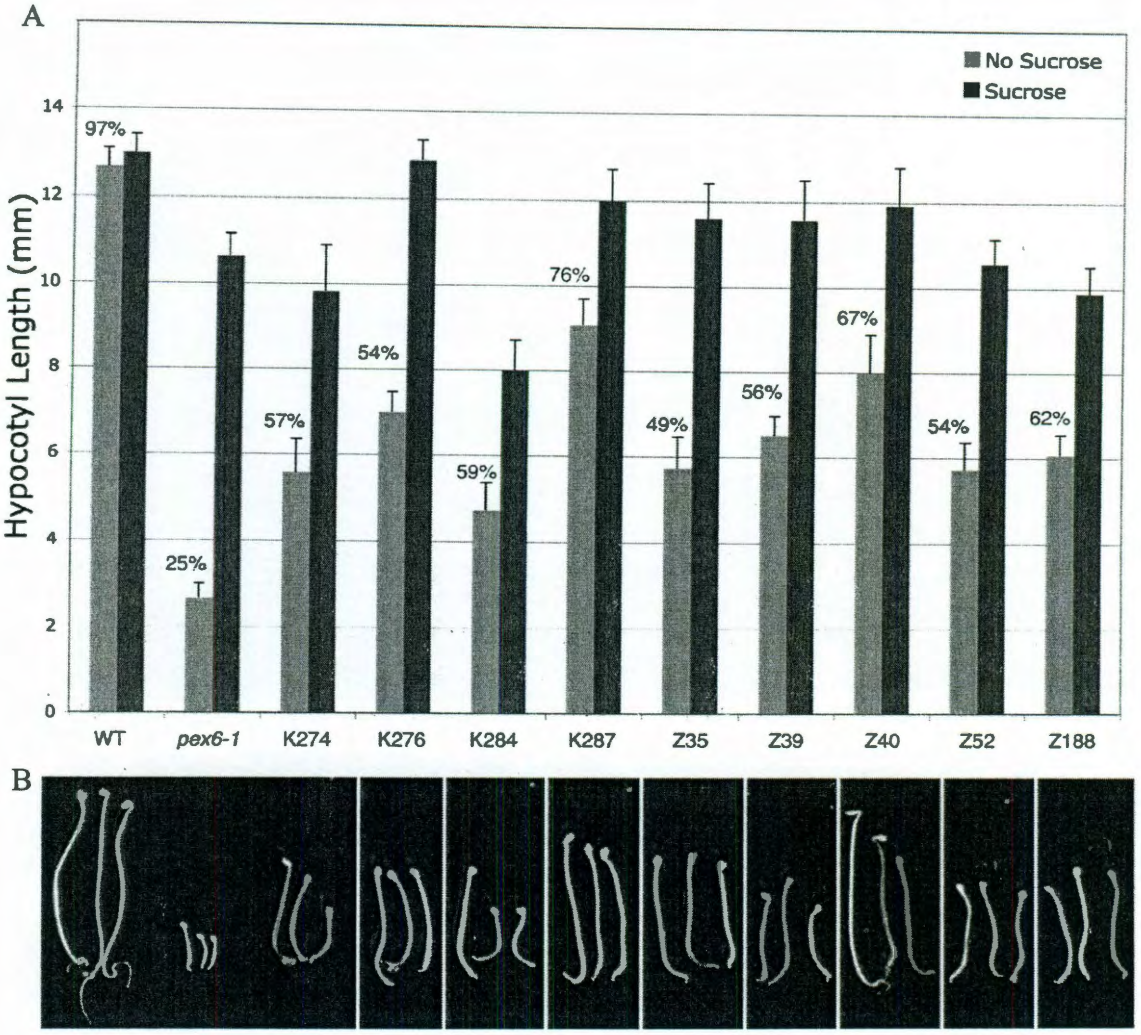

Figure B.1 Sucrose dependence of *pex6-1* modifiers.
A. All modifiers show intermediate sucrose dependence compared to wild type and *pex6-1*. Seeds were plated on medium lacking or containing 20 mM sucrose. Hypocotyls were measured after 1 day of light and 4 days dark. Error bars indicate standard errors of the means ($n \geq 12$). Numbers above the bars indicate the percentage of growth on no sucrose versus growth on sucrose. B. Three representative seedlings taken from the no sucrose plate in A from each modifier line are shown.

(K modifiers) did not show greater levels of sucrose independence than modifiers isolated for enhanced IBA resistance (Z modifiers).

IBA resistance was also variable in the modifiers (Figure B.2). Wild-type root elongation is significantly inhibited on 15 μM IBA, whereas *pex6-1* roots are less sensitive to IBA. Of the modifiers isolated in either screen, Z188 was the only modifier displaying less IBA resistance then *pex6-1*, suggesting the possibility of a slight suppression of *pex6-1* IBA resistance. Several modifiers had similar inhibition of growth as *pex6-1* when grown on IBA including K284, K276, K274, Z40, and Z52. Lastly, K287, Z35, and Z39 appear to be slightly more IBA resistant than *pex6-1*.

To gain insight into the suppression mechanism of the *pex6-1* modifiers, I analyzed PTS2 processing via Western blot in the mutagenized lines. Both wild-type accessions, Columbia and Ws, have completely processed thiolase as opposed to *pex6-1*, in which thiolase processing was defective (Figure B.3). Of the nine *pex6-1* modifier lines, only Z40 and Z52 had improved thiolase processing compared to *pex6-1*, while the remaining seven were similar to *pex6-1* (Figure B.3). This result indicates that there are at least two classes of *pex6-1* modifiers. The Z40 and Z52 defect may restore the *pex6-1* peroxisomal defect, whereas the other modifiers may bypass the *pex6-1* defect.

To identify the modifiers, I began mapping in outcrossed lines using Ws as the alternative ecotype because Bethany Zolman originally mapped *pex6-1* by crossing to the Ws background using IBA resistance to define her mapping population (Zolman and Bartel, 2004). However, despite sizable mapping populations, I was unable to isolate a mapping interval in Z40, Z188, and K274. One explanation for the lack of a mapping interval in all three lines could be that another suppressor may have entered the mapping

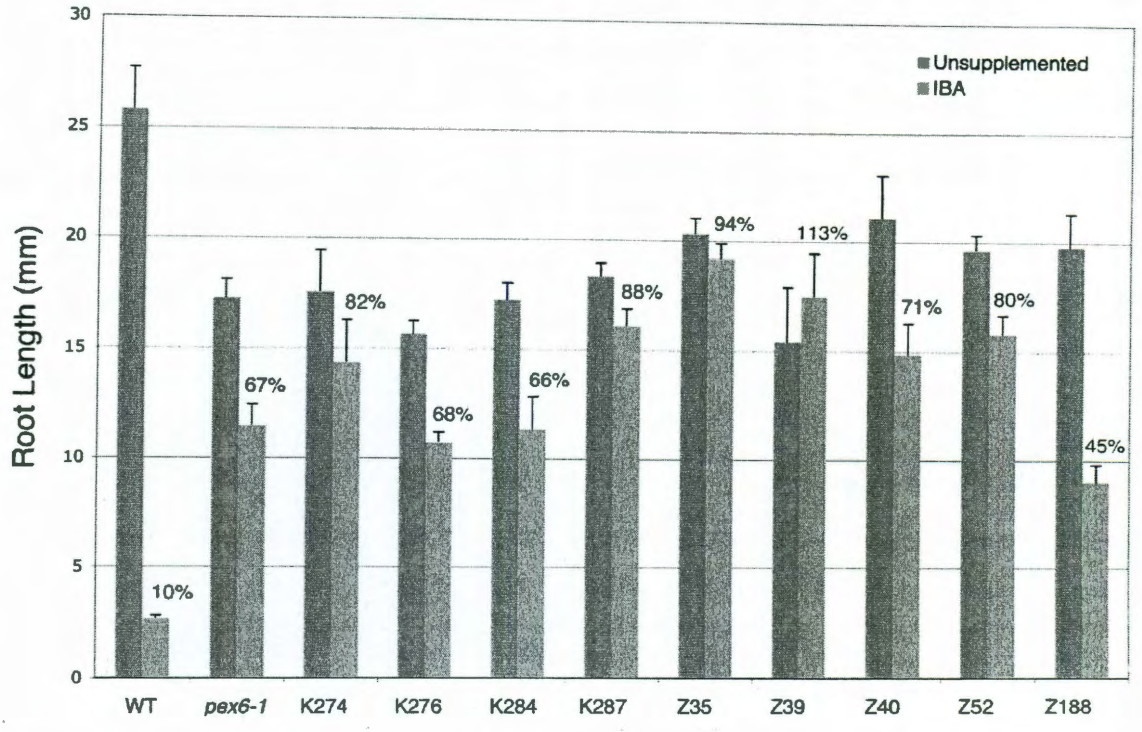

Figure B.2 *pex6-1* modifiers remain IBA resistant when compared with wild type. Seeds were plated on plant medium with and without 15 μM IBA, incubated under yellow light for 7 days, and root lengths were measured. Error bars indicate standard errors of the means ($n \geq 12$). Numbers above the IBA bar indicate the percentage of root growth on IBA versus unsupplemented medium.

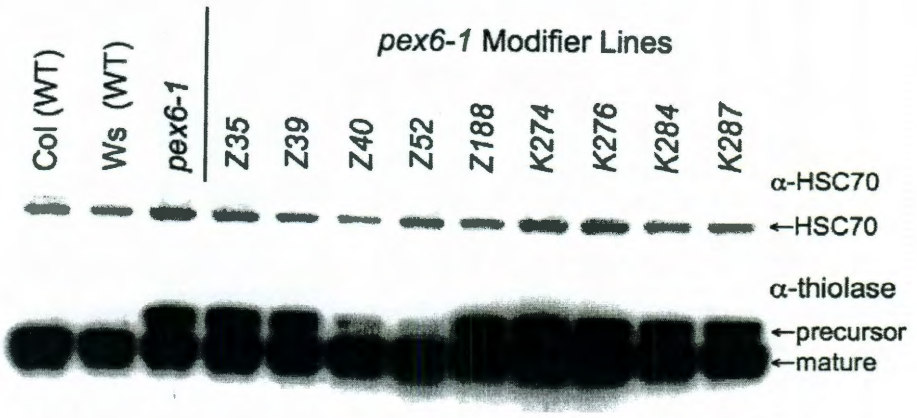

Figure B.3 *pex6-1* thiolase-processing defect is partially restored in a subset of modifiers.
Protein samples from eight 3-day-old seedlings grown on plant medium with sucrose were immunoblotted using α-thiolase. The top bands, precursor, represent unprocessed thiolase and the bottom bands, mature, represents processed thiolase. α-HSC70 was used as a loading control.

population from the Ws ecotype skewing the possibility of finding a single genomic region that is responsible for the acquired sucrose independence of *pex6-1*.

To determine if Ws had an accession-specific suppressor of *pex6-1*, I introgressed *pex6-1* from the Columbia background to the Ws background. To create the *pex6-1* introgression line, *pex6-1* was crossed into Ws and the IBA resistant progeny was repeatedly outcrossed to Ws. PCR-based markers were used to detect the content of Ws polymorphisms on each chromosome and the line with the closest proximity of Ws markers to *pex6-1* was used. IBA resistance is the most efficient phenotype to follow for isolating homozygous *pex6-1* seedlings because this trait is recessive and does not require a recovery period after treatment (unlike sucrose dependence assays). Therefore, IBA resistance was used to follow *pex6-1* during introgression.

After introgressing *pex6-1* into Ws, I performed a sucrose dependence assay in the dark to determine if a suppressor from the Ws background could confer sucrose independence in combination with the *pex6-1* lesion. Indeed, *pex6-1* introgressed into Ws is sucrose independent (Figure B.4), indicating that the Ws background harbors at least one region that suppress *pex6-1*. Because of this suppressor, Ws was an unsuitable accession to outcross for mapping.

To determine if the Ws modifier is a general *pex* modifier or if it is specific to *pex6-1*, I also examined the sucrose dependence of two *pex4-1* introgression lines. When grown on medium that lacks sucrose, the introgression lines 1A, 1B, and 4A are unable to elongate their hypocotyls in the dark similarly to *pex4-1* (Figure B.5). However, some seedlings have hypocotyl lengths that exceed the standard deviation, suggesting that there could be a gene modifying *pex4-1* in Ws that is not yet homozygous for the Ws allele.

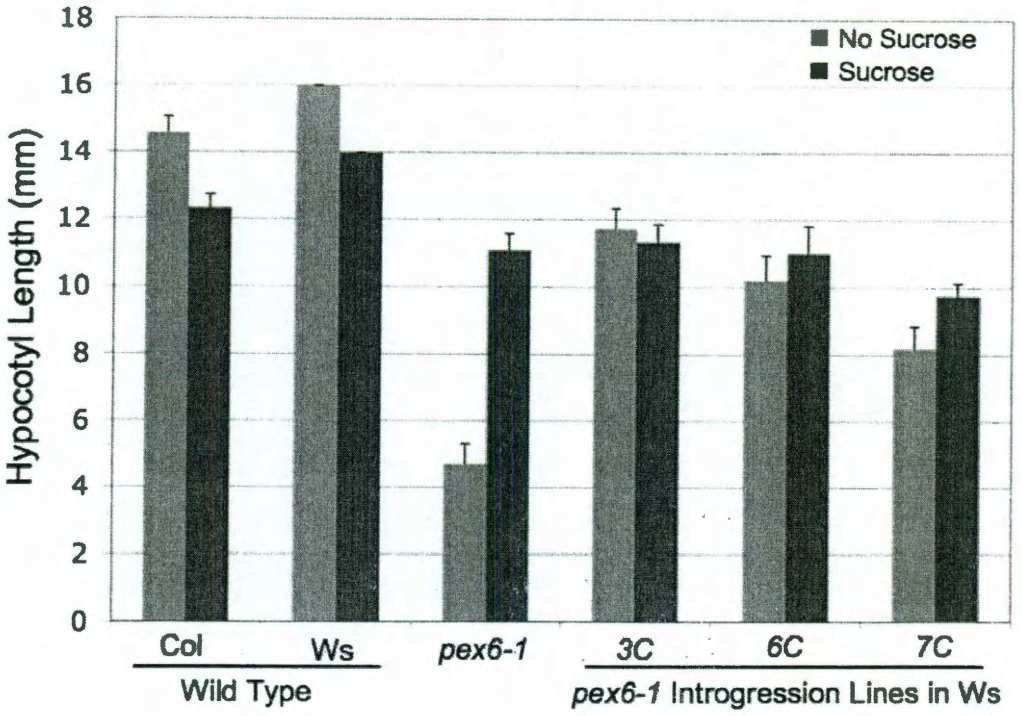

Figure B.4 *pex6-1* is not sucrose dependent in the Ws accession.
Col and Ws are wild type accessions, *pex6-1* is in the Col background, *pex6-1* introgression lines are from the third outcross of *pex6-1* to Ws. Seeds were plated on medium lacking or containing 20 mM sucrose. Hypocotyls were measured after 1 day of light and 4 days of dark growth. Error bars indicate standard error of the means ($n \geq 10$).

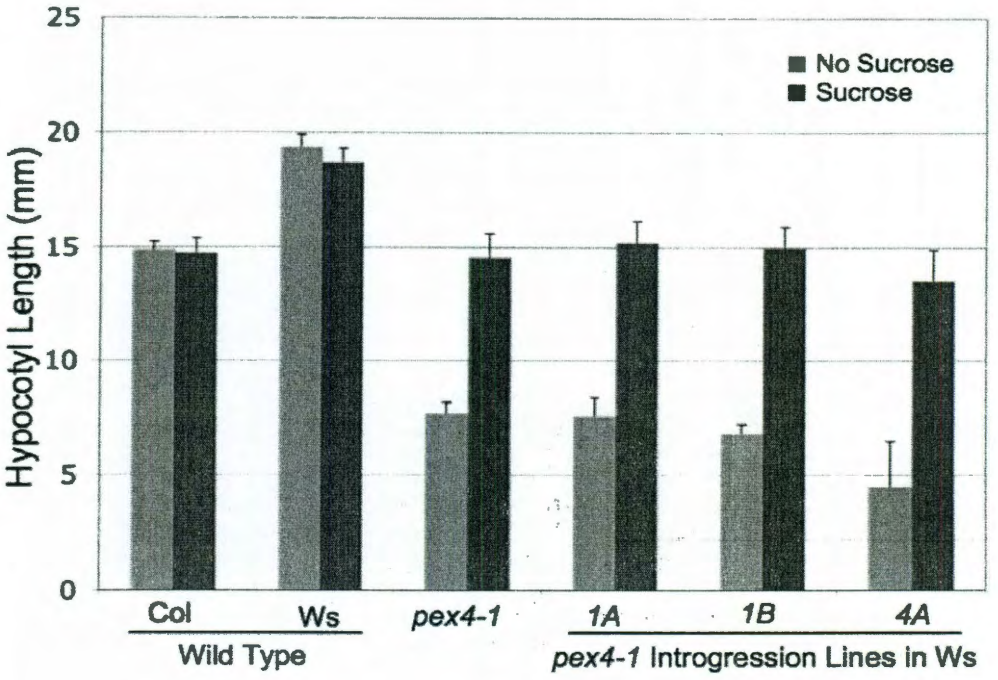

Figure B.5 *pex4-1* remains sucrose dependent in the Ws accession.
Col and Ws are wild type accessions, *pex4-1* is in the Col background, *pex4-1* introgression lines are from the third outcross of *pex4-1* to Ws. Seeds were plated on medium lacking or containing 20 mM sucrose. Hypocotyls were measured after 1 day of light and 4 days of dark growth. Error bars indicate standard error of the means ($n \geq 10$).

Landsberg (L*er*) was the first alternative accession considered for generating new mapping crosses once the Ws modifier was discovered, because sucrose-dependent peroxisomal mutants, such as *chy1*, *ped1*, *ped2*, and *ped3/pxa1* (Zolman et al., 2001a; Hayashi et al., 1998; Zolman et al., 2001b), have been isolated in the Ler background, suggesting the lack of a general *pex* suppressor in Ler. In addition, Ler is a widely used accession and has many published polymorphisms to utilize for mapping. To determine if Ler would be an acceptable mapping background for the *pex6-1* modifiers, I crossed *pex6-1* to Ler and tested two segregating F2 lines for sucrose dependence. I found no homozygous *pex6-1* seedlings that were sucrose independent, suggesting that there doesn't appear to be an accession-specific *pex6* modifier in Ler (Figure B.6).

After confirming that Ler appears to be an acceptable mapping background, I made new mapping crosses for each of the *pex6-1* modifiers. After testing several mapping plant isolation methods, I determined the strategy most effective at isolating mapping plants is a two-generation approach in which F2 plants are selected based on IBA resistance and the F3 progeny are screened for sucrose independence. This method was particularly effective at enriching the *pex6-1* plants. Progeny testing was necessary to determine the genotypic state of the modifier in each mapping plant and would be required regardless of the necessity to define the mapping population. Despite the outcross to Ler and repeated mapping attempts, I was unable to define a mapping interval in any of these lines.

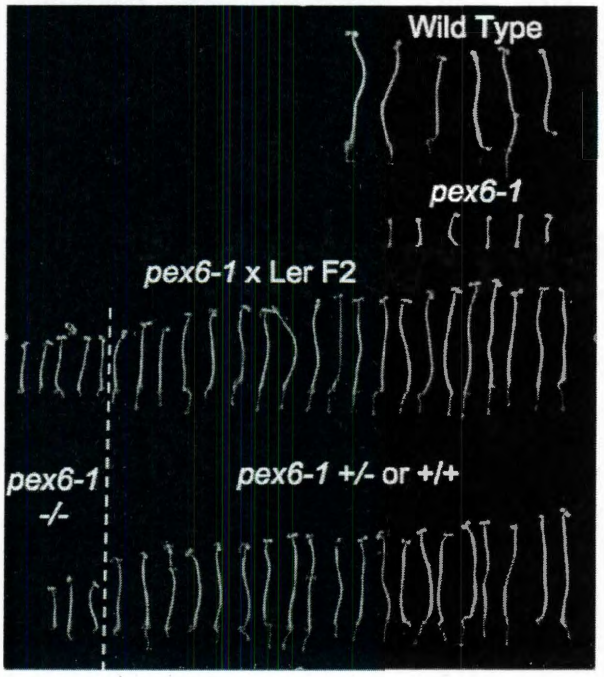

Figure B.6 *pex6-1* sucrose dependence is not suppressed in the L*er* accession. Seeds were plated on plant medium lacking sucrose and incubated for 1 day in the light followed by growth for 4 days in the dark. In the bottom two rows, *pex6-1* x Ler F2 seedlings were arranged by hypocotyl length, photographed, and genotyped for *pex6-1*. No *pex6-1* homozgyotes were found amoung the sucrose-independent seedlings.

C. Differential PTS1 and PTS2 import defects reveal a distinct role for Arabidopsis PEX14 in the PTS1 pathway of peroxisomal matrix protein import

Monroe-Augustus, M., Ramon, N.M., Ratzel, S.E., Lingard, M.J., Murali, C., and Bartel, B. (2010) Differential PTS1 and PTS2 import defects reveal a distinct role for *Arabidopsis* PEX14 in the PTS1 pathway of peroxisomal matrix protein import. *In revision.*

D. Mutation of *E1-CONJUGATING ENZYME-RELATED1* Decreases *RELATED TO UBIQUITIN* Conjugation and Alters Auxin Response and Development

Woodward, A.W., Ratzel, S.E., Woodward, E.E., Shamoo, Y., Bartel, B. (2007) Mutation of *E1-CONJUGATING ENZYME-RELATED1* Decreases *RELATED TO UBIQUITIN* Conjugation and Alters Auxin Response and Development. Plant Physiology 144(2): 976-987.